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DISSERTATION

Opioids in Neuropathic Pain
The Role of Potassium Channels in Peripheral Sensory Neurons

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité - Universitätsmedizin Berlin

von

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aus Berlin

Datum der Promotion:

16.06.2018

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Abstract

Neuropathic pain is a debilitating condition often arising from damage to peripheral nerves. Patients suffer from spontaneous pain and pain evoked by thermal / mechanical stimuli. The underlying pathophysiology is complex and poorly understood. Opioids are powerful analgesics; however, their use is limited by centrally mediated side effects like respiratory depression and addiction. Activation of opioid receptors in peripheral sensory neurons is devoid of adverse centrally induced effects and can ameliorate neuropathic pain in animal models. Mechanisms of peripheral opioid analgesia have therefore gained interest in pain research. Among the proposed mediators are G protein-coupled inwardly-rectifying potassium ($K_{ir}3$) channels. However, conflicting evidence has been presented on $K_{ir}3$ channel contribution to peripheral opioid analgesia.

The goal of this study was to investigate changes in opioid mediated potassium conductance upon peripheral nerve injury in mice. We hypothesized that a lesion to a peripheral nerve results in enhanced opioid-induced potassium conductance through $K_{ir}3$ channels in the corresponding sensory neurons. To mimic neuropathic pain, a chronic constriction injury (CCI) was used. To examine the effects of CCI on potassium channel conductance mediated by the selective μ-opioid receptor agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), potassium imaging and patch clamp were employed. Recordings were carried out in cultured dorsal root ganglia (DRG) neurons from mice without (naïve) and with CCI. For verification, similar experiments were performed in transfected human embryonic kidney (HEK) 293 cells.

Using patch clamp, I successfully measured DAMGO-induced potassium currents, reversible by barium chloride, in μ-opioid receptor and $K_{ir}3.2$ expressing HEK 293 cells as well as in DRG neurons from naïve and CCI mice. Contrary to our assumption, the DAMGO-induced potassium currents were significantly smaller in neurons from CCI mice compared to naïve mice. However, CCI did not change the rate of DAMGO-responders compared to naïve neurons. Potassium imaging experiments in HEK 293 cells and mouse DRG neurons did not provide conclusive data and raised methodological concerns regarding reliability and validity of the assay.

These results confirm the opening of potassium channels by DAMGO in DRG neurons of naïve and CCI mice. They don't support the hypothesis that CCI results in enhanced K_{ir} channel conductance upon application of DAMGO in DRG neurons, but rather show a tendency towards smaller currents after CCI.

This thesis provides new insight into the interaction of potassium channels and opioid receptors on murine peripheral sensory neurons in health and neuropathy, and important methodological considerations for further investigations into the site-specific contributions of potassium channels to peripheral opioid analgesia in neuropathic pain.

Zusammenfassung

Neuropathische Schmerzen sind beeinträchtigende Veränderungen der Sensibilität, welche oft durch Läsionen peripherer Nerven verursacht werden. Patienten leiden u.a. an Spontanschmerz und Schmerz durch Temperatur- und Berührungsreize. Die zugrundeliegende Pathophysiologie ist komplex und wenig verstanden. Opioide sind wirkungsvolle Schmerzmittel, deren Einsatz jedoch durch zentralnervöse Nebenwirkungen wie Atemdepression und Abhängigkeit limitiert ist. Die Aktivierung von Opioidrezeptoren auf peripheren sensorischen Neuronen ist frei von diesen Nebenwirkungen und lindert neuropathische Schmerzen in Tiermodellen. Mechanismen peripherer Opioidanalgesie erfahren daher vermehrtes Interesse in der Schmerzforschung. Zu den vorgeschlagenen Mediatoren peripherer Opioidanalgesie gehören G-Protein-gekoppelte einwärtsgleichrichtende Kaliumkanäle ($K_{ir}3$). Die Studienlage bezüglich der Rolle von $K_{ir}3$ in peripherer Opioidanalgesie bei neuropathischen Schmerzen ist bislang jedoch nicht eindeutig.

Das Ziel der vorliegenden Arbeit war, Veränderungen der opioidinduzierten Kaliumleitfähigkeit nach peripherer Nervenläsion bei Mäusen zu untersuchen. Wir nahmen an, dass die Läsion eines peripheren Nerven eine gesteigerte opioidinduzierten Kaliumleitfähigkeit durch $K_{ir}3$ Kanäle in den entsprechenden Neuronen zur Folge hat. Als Modell für neurophatischen Schmerz verwendeten wir eine chronic constriction injury (CCI). Die Wirkungen der CCI auf die durch den μ-Opioidrezeptoragonisten [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) ausgelöste Kaliumleitfähigkeit untersuchten wir in kultivierten Neuronen aus Spinalganglien von Mäusen mit und ohne CCI mit Hilfe von Potassium Imaging und Patch Clamp. Zusätzlich verwendeten wir transfizierte human embryonic kidney (HEK) 293 Zellen.

Mit Hilfe von elektrophysiologischen Methoden konnte ich erfolgreich DAMGO-induzierbare Kaliumströme in MOR und $K_{ir}3.2$ exprimierenden HEK 293 Zellen sowie peripheren Neuronen von Mäusen mit und ohne CCI messen, welche durch $BaCl_2$ reversibel waren. Im Gegensatz zu unserer Hypothese waren die gemessenen Kaliumströme in CCI-Neuronen kleiner als in Neuronen naiver Mäuse. CCI veränderte jedoch die Rate der DAMGO-Antworten verglichen mit der naiven Gruppe nicht. Die Potassium-Imaging-Experimente an HEK 293 Zellen und Maus-Neuronen erbrachten keine schlüssigen Ergebnisse und warfen Fragen bezüglich Reliabilität und Validität des verwendeten Assays auf.

Diese Ergebnisse bestätigen die Öffnung von Kaliumkanälen durch DAMGO in primären afferenten Neuronen von naiven Mäusen und Mäusen nach CCI. Die Hypothese, dass CCI zu erhöhter DAMGO-induzierbarer Leitfähigkeit von $K_{ir}3$-Kanälen in primären afferenten Neuronen führt, wurde nicht bestätigt. Die reduzierte Opioidwirkung auf Kaliumströme nach CCI könnte auf Umverteilung von Kaliumkanälen und/oder μ-Opioidrezeptoren von Zellkörpern peripherer Neurone hin zum Ort des CCI am Axon hinweisen.

Die vorliegende Arbeit liefert neue Erkenntnisse zur Interaktion von Opioidrezeptoren und Kaliumkanälen in peripheren Neuronen der Maus in Gesundheit und unter Neuropathie sowie wertvolle methodologische Hinweise für weitere Untersuchungen der Rolle subzellulärer Lokalisation von Kaliumkanälen für Opioidanalgesie bei neuropathischen Schmerzen.

Abbreviations

A	ampere
ANOVA	analysis of variance
ASIC	acid-sensing ion channel
ATP	adenosine triphosphate
AUC	area under the curve
Ba	barium
Ca	calcium
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
Cl	chloride
CNS	central nervous system
CO_2	carbon dioxide
DAG	diacylglycerol
DAMGO	[D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin
DMEM	Dulbecco's modified eagle medium
DRG	dorsal root ganglion
E_K	equilibrium potential for K^+
E_{rest}	resting membrane potential
GIRK	G protein-coupled inwardly rectifying potassium channel = $K_{ir}3.x$
GPCR	G protein-coupled receptor
H	hydrogen
h	hour
HEK 293	human embryonic kidney 293 cells
HEPES	hydroxyethyl piperazineethanesulfonic acid
HIV	human immunodeficiency virus
Hz	hertz
IASP	International Association for the Study of Pain
IP_3	inositol trisphosphate
K	potassium
K_{2P}	tandem pore domain potassium channel
K_{ATP}	ATP-regulated potassium channel
K_{Ca}	calcium-activated potassium channel
KCNJ3	potassium inwardly-rectifying channel, subfamily J, member 3 = $K_{ir}3.1$

K_{ir}	inwardly rectifying potassium channel
K_v	voltage-gated potassium channel
L	lumbar
m	meter
M	mol
Mg	magnesium
min	minute
MOR	μ-opioid receptor
mRNA	messenger ribonucleic acid
Na	sodium
NICE	National Institute for Health and Care Excellence
OR	opioid receptor
PBS	phosphate buffered saline
PIP_2	phosphatidylinositol 4,5-bisphosphate
ROI	region of interest
s	second
SEM	standard error of mean
SUR	sulfonylurea receptor
TEA	tetraethylammonium chloride
TG	trigeminal ganglion
TREK	potassium two pore domain channel subfamily K
TRESK	TWIK-related spinal cord potassium channel
TRPM8	receptor potential cation channel subfamily M member 8
TRPV1	transient receptor potential cation channel subfamily V member 1
V	volt
VZV	varicella zoster virus

1. Introduction

"Reckoning with pain, in its acute form, is an essential aspect of human existence," writes Malcolm Barrett in his literature review on pain (Barrett, 2013). Indeed, acute pain can be a warning sign, which makes us aware of (potential) harm. It impacts our behavior helping to prevent further tissue damage and thereby fostering recovery. Pain is often the most important clue, physicians look for to identify disease or impending tissue damage. Suffering pain fundamentally influences our emotional wellbeing and capacity to participate in society (Buytendijk and Plessner, 1948).

Thus, Melzack and Casey described pain as a multidimensional and complex phenomenon, with sensory-discriminative, affective-motivational and cognitive-evaluative components (Melzack and Casey, 1968). Currently, the International Association for the Study of Pain defines pain as 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, 2012). While this definition has proven fruitful for pain research for a long time, it is worth stressing that pain always "occurs in context of the individual and the sociocultural factors that mold its experience and display" (Barrett, 2013).

Acute pain usually does not challenge our ability to rationally attribute salience to its existence. We are taught to perceive it as temporary and even helpful, thus, merely await its fading away. With its existential integration into our functioning and wellbeing, however, it is easily understandable that chronic pain, which does not subside, challenges our ability to provide salient explanations for its existence (Cervero, 2014). Chronic pain in this sense can develop into a disease of its own, reshaping body and mind (Grüny, 2004).

Treatment of chronic pain is often challenging and the lack of successful innovative therapies in the last decades has led to frustration among patients and doctors (Woolf, 2010). Occurrence of side effects often restricts the use of otherwise beneficial pain medications. Among these are opioids such as morphine and fentanyl, which are considered the most powerful analgesics available (Zöllner and Stein, 2007). Nevertheless, serious side effects, including respiratory depression, sedation, dizziness, nausea, and addiction, mediated by opioid receptors in the brain, limit the use of opioids in clinical practice. Interestingly, activation of opioid receptors outside the brain, in peripheral tissues such as skin, muscles, and joints, can provide analgesia without centrally-mediated adverse effects. Among the suggested mechanisms underlying peripheral opioid analgesia is activation of potassium channels in peripheral sensory neurons (Stein and

Machelska, 2011). Hence, interactions of opioids with potassium channels pose a promising framework for the development of novel therapeutic strategies with improved side effect profile.

This study investigates the effects of opioids on potassium channel conductance in the peripheral sensory neurons under neuropathic pain conditions.

1.1. Pain and Nociception

1.1.1. Anatomy of Pain Pathways

Perception of bodily sensations in humans and other mammals is based on activation of peripheral nerve cells which convey sensory information from the site of stimulation to the central nervous system (CNS). In the CNS, sensory input of different modalities is modulated and integrated with other information on the state of the organism and its environment to finally be perceived as touch, temperature, vibration or pain (Woolf and Salter, 2000).

Primary sensory neurons transmit somatosensory information from peripheral tissues such as skin, joints, muscles and viscera to the spinal cord and the brain. Their cell bodies are situated in the trigeminal ganglia and dorsal root ganglia (DRG) and their morphology is commonly described as pseudo-unipolar, i.e. one common axo-dendritic process, originating from the cell body, bifurcates into a central and a peripheral process forming one axo-dendritic conduction pathway. The peripheral terminals of primary sensory neurons innervate peripheral tissues, whereas the central terminals enter the spinal cord or the brain stem and synapse to secondary sensory neurons, interneurons and neurons of descending pathways (see Figure 1.1).

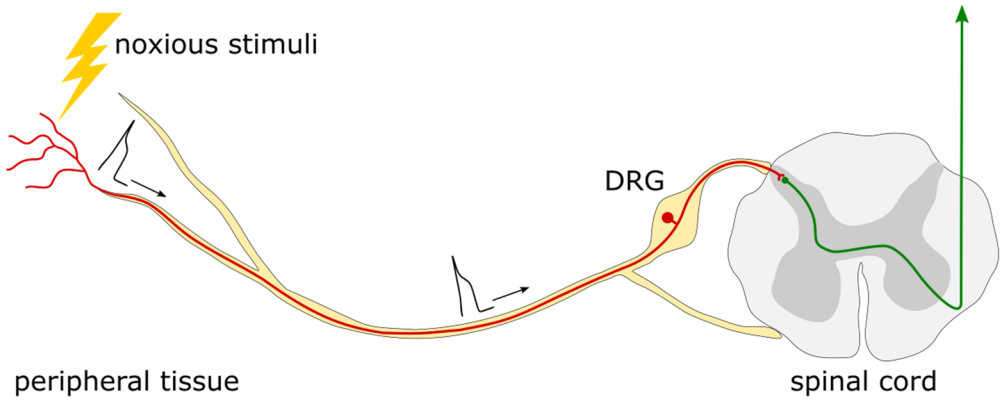

Figure 1.1: Pain pathway. Peripheral nerve (yellow) with primary afferent neuron (red) synapsing to secondary afferent neuron (green) in the dorsal horn of the spinal cord. Noxious stimuli in peripheral tissue generate action potentials, which are then propagated along the axon towards the central terminal (modified after Waxman and Zamponi, 2014).

Axons of primary afferent neurons are enveloped by Schwann cells producing myelin sheaths (myelinated fibers) or bare cytoplasmic processes of Schwann cells (unmyelinated fibers). Hence, primary afferent neurons are usually classified by the degree of myelination as well as size, conduction velocity, input threshold, and sensory modality. Accordingly, they can be divided into Aβ, Aδ, and C-fiber neurons. Aβ-fiber neurons have the largest diameter and thickly myelinated axons, are fast conducting, and terminate in specialized receptive structures such as Merkel cells or Ruffini corpuscles in the skin. Typically, they have a low activation threshold and convey non-painful touch sensations under physiological conditions, but can transmit pain in pathological states. Aδ-fiber neurons are medium sized and thinly myelinated axons of slower conductance velocity, terminate in peripheral tissues as free nerve endings, have high activation threshold and convey painful mechanical stimuli. C-fiber neurons have unmyelinated, small diameter axons, are slowly conducting, terminate as free nerve endings, have high activation threshold and convey painful mechanical, heat, cold, and chemical stimuli (Basbaum et al., 2009).

1.1.2. Molecular Mechanisms of Nociception

To reach the spinal cord and ultimately the brain, the painful stimulus first needs to be transformed into an electro-chemical signal in the primary afferent neuron. This process is called transduction and is mediated by specialized membrane proteins that respond to their respective stimulus. These proteins comprise ion channels sensitive to heat, for example, transient receptor potential cation channel subfamily V member 1 (TRPV1), cold, like transient receptor potential cation channel subfamily M member 8 (TRPM8), acid, such as acid-sensing ion channels (ASICs), and receptors specific for a variety of mediators, including proinflammatory cytokines such as interleukin-1β and tumor necrosis factor (Woolf and Ma, 2007). Due to the expression of various of these ion channels and receptors, most nociceptive neurons, particularly C-fiber neurons, are involved in encoding several modalities of painful sensation and are thus called “polymodal” sensors. Activation of transducer channels (such as TRPV1, TRPM8, ASICs) leads to depolarization of the cell membrane and activation of voltage-gated ion channels, which culminates in the generation and propagation of actions potentials (APs) along the axonal membrane of the primary afferent neuron (Figure 1.1). Once an AP reaches the central terminal in the CNS, it facilitates calcium dependent release of neurotransmitters, like glutamate or substance P, from vesicles stored at the pre-synaptic membrane into the synaptic gap (Scholz and Woolf, 2002). All these processes, i.e. signal

transduction, AP generation and propagation as well as transmitter release, are dependent on a complex interplay of a variety of ion channels. Excitatory mechanisms primarily rely on sodium and calcium influx, leading to depolarization of the membrane at the site of stimulation. In contrast, opening of potassium and chloride channels generally produces opposite response leading to repolarization or hyperpolarization of the membrane, which counteracts the excitatory impulse (Basbaum et al., 2009).

The above paragraphs mainly describe the process known as nociception, a term coined by C. Sherrington in 1906 to distinguish the mere physiological response of the nervous system to a noxious stimulus from the subjective experience of pain (Sherrington, 1906). Perception of pain is a more complex phenomenon involving peripheral, spinal as well as supra-spinal integration of internal bodily, environmental as well as cognitive factors. For a historical overview of conceptualizations of pain perception see Moayedi and Davis, 2013.

1.1.3. Chronic Pain

"By chronic pain I mean pain that is not endured for some purpose or goal […], pain that promises to go on indefinitely (although sometimes intermittently and sometimes unpredictably), pain that demands no action because as far as we know, no action can get rid of it." (Wendell, 1996)

Acute pain, resulting from transient insults to peripheral tissues, usually presents as short-lasting pain which resolves with cessation of the noxious stimulus. This kind of pain is generally considered a warning mechanism, which protects the organism from harm or helps it to recover from injury. Management of acute pain usually does not present a major challenge to clinicians or their patient.

Persistent pain however, which results from permanent tissue damage, including mechanical injury, persistent inflammation, or metabolic imbalance, leads to long-lasting pathological changes in the pain pathways on molecular and cellular levels. These mal-adaptive states can persist long after cessation of the initial noxious stimulus and present a major challenge to clinicians and their patients. Chronic pain is thus now increasingly considered an entity of its own, which demands understanding of underlying mechanisms and development of specific therapeutic approaches (Woolf and Salter, 2000).

Epidemiology

Chronic pain is a widespread problem in our societies. In European countries, 12 – 30% of adults suffer from chronic pain.[1] In Germany, 17% of the population experience chronic pain and a third of them is severely limited in managing their activities of daily life (Breivik et al., 2006). Besides the relevance for the individual's quality of life, chronic pain is a major economic challenge for societies and their social and health care systems. It is estimated to cause costs of several billion Euro each year in Germany, mainly due to temporary or permanent disability and early retirement (Deutsche Gesellschaft zum Studium des Schmerzes (DGSS), 2006).

1.2. Neuropathic Pain

Among the different forms of chronic pain, neuropathic pain is particularly disabling and difficult to treat. Due to its multifaceted patterns of presentation, a commonly used definition of neuropathic pain in research relies on etiology rather than specific symptoms:

"*Neuropathic pain is pain caused by a lesion or disease of the somatosensory nervous system*"

(International Association for the Study of Pain, 2012).

In the clinical setting, however, neuropathic pain is generally diagnosed by the clinical presentation rather than the often unknown etiology. It is typically characterized by unpleasant changes in certain aspects of sensory perception in the affected regions of the body. Patients experience reduced thresholds to painful mechanical or thermal stimuli (hyperalgesia), or pain sensations elicited by normally innocuous stimuli like gentle touch, warm or cool temperatures (allodynia). Another frequently reported feature is spontaneous, externally unprovoked pain, described as shooting, electrifying, or burning. Additionally, patients also report non-painful, but equally unpleasant, symptoms such as loss of sensitivity (hypoesthesia) or unusual perceptions, commonly reported as pins and needles (paresthesia) (Baron et al., 2010).

Neuropathic pain can develop following lesions to the CNS (spinal cord injury, stroke, and multiple sclerosis) or to peripheral nerves. Here, I will focus on the latter. Peripheral neuropathic pain can be caused by a variety of diseases and injuries. Among the most common causes are diabetic polyneuropathy, herniated vertebral discs, tumor, or chemotherapy,

[1] In this study, chronic pain was defined as pain lasting more than 6 months, having an intensity of 5 or more on a Numeric Rating Scale: 1 (no pain) to 10 (worst pain imaginable), Breivik et al. (2006).

infectious agents such as human immunodeficiency virus or varicella zoster virus or injuries to nerves due to accidents or medical procedures (Jensen and Finnerup, 2014). The patterns of distribution differ between neuropathies: Whereas diabetic or alcoholic neuropathy usually present as a polyneuropathy in a symmetric “glove and stocking” pattern on hands and feet, traumatic neuropathies following bruising or disruption of peripheral nerves or post-herpetic neuralgia might present in a single localized area supplied by the affected nerve. This initially affected area then often extends over time producing an “aura” of pain around the initial territory of altered sensation (Campbell and Meyer, 2006).

Mechanisms responsible for the development of neuropathic pain are diverse, including ectopic impulse generation (in cell bodies of affected neurons or at the site of nerve injury), degeneration or sprouting of sensory fibers as well as sympathetic nerves into areas normally not innervated by these fibers. These events are associated with alterations in transmitter synthesis and expression as well as signaling of receptors and ion channels, which spawn the key feature all neuropathic pain states share: reduced activation threshold of primary afferent neurons, also called sensitization.

On a molecular level, this peripheral sensitization involves changes in transducer channels such as TRPV1 or TREK-1, which lead to bigger generator potentials to the same stimulus intensity than under healthy conditions, facilitating action potential generation in the membrane of peripheral terminals. Additional changes occur in the apparatus generating action potentials, i.e. mainly voltage-gated sodium channels responsible for depolarizing the membrane beyond spike generation threshold and potassium channels responsible for stabilizing the membrane potential and modulating AP duration (Waxman and Zamponi, 2014).

Ultimately, these alterations lead to substantially reduced activation thresholds of peripheral afferents resulting in spontaneous pain, hyperalgesia, and allodynia following nerve damage (Scholz and Woolf, 2002; Baron et al., 2010).

This thesis focuses on potassium channels in the regulation of excitability of peripheral afferents in neuropathic pain.

1.3. Treatment of Neuropathic Pain

„If there is a single experience shared by virtually all chronic pain patients it is that at some point those around them - chiefly practitioners, but also at times family members - come to question the authenticity of the patient's experience of pain.“ (Kleinman, 1988)

The described experience of questioned authenticity might be particularly relevant for patients suffering from neuropathic pain conditions, since symptoms can fluctuate and be difficult to describe to relatives and doctors. With the estimated population prevalence of pain with neuropathic characteristics in Europe and North America ranging from 6.9% to 12% (van Hecke et al., 2014; Yawn et al., 2009), treatment of neuropathic pain presents a major challenge to clinicians in many areas of medicine such as diabetology, neurology, and oncology.

This is aggravated by the fact that many patients do not experience sufficient symptom control or pain relief (Baron et al., 2010; Finnerup et al., 2010). Sleep disturbances, depression and anxiety are frequently encountered comorbidities, which need to be taken into account when planning and evaluating the treatment success of painful neuropathies (Baron et al., 2010).

Various pharmacological approaches have been used to counteract neuropathic symptoms. While no major innovations have been presented in the field during the last years, treatment recommendations keep changing due to reevaluation of efficacy and side effect profiles. Recent guidelines recommend antidepressants or anticonvulsants as first line treatment for all neuropathic pain conditions (Finnerup et al., 2015; National Institute for Health and Care Excellence (NICE), 2014). Only after trying more than one of the substances considered first line, the synthetic opioid tramadol or local treatments with lidocaine or capsaicin patches are recommended (National Institute for Health and Care Excellence (NICE), 2014). Nonsteroidal anti-inflammatory drugs are no longer recommended for treating neuropathic pain due to their insufficient effectiveness in symptom relief (Deutsche Gesellschaft für Neurologie, 2012).

Nevertheless, all currently used medications produce numerous side effects which limit their analgesic efficacy. Tricyclic antidepressants have pronounced anticholinergic properties, which advise cautious use especially in the elderly and patients with heart conditions. Dry mouth, constipation and blurred vision are frequent causes for termination of treatment by patients. Serotonin–norepinephrine reuptake inhibitors, while less anticholinergic, can give rise to orthostatic dysregulation, nausea and sexual dysfunction (McQuay, 2002). Anticonvulsants such as gabapentin or pregabalin frequently show central side effects such as dizziness,

somnolence or disturbance in gait, cautioning careful dose titration and monitoring, especially in the elderly (McQuay, 2002).

1.3.1. Opioids in the Treatment of Neuropathic Pain

Opioids have long been used and still remain the most powerful analgesics to treat severe acute and chronic pain (Stein and Lang, 2009). Evidence for use of opium, today known to contain several analgesic opioid alkaloids, dates back as far as to the neolitic age (Merlin, 2003). First isolated in 1803 by German pharmacist F. W. Sertürner, morphine, the most prominent among the opioids, gained widespread commercial use since the 1820s (Sertürner, 1805; International Association for the Study of Pain, 2010).

Whereas opioids show beneficial analgesic effects in the treatment of neuropathic pain (McNicol et al., 2013), higher doses are needed than in other conditions, which increases the risk for side effects (Reinecke et al., 2014; Zöllner and Stein, 2007). Side effects frequently encountered by patients treated with opioids include constipation, nausea and sedation. Although they have long been considered second or even first line in neuropathic pain treatment, they are now listed as third line medications (Binder and Baron, 2016), primarily due to concerns regarding risk of abuse and safety (Finnerup et al., 2015). In light of the so called 'opioid epidemic' in the USA (Paulozzi, 2012; Manchikanti et al., 2012) the additional risk of addiction and overdosing-related deaths have recently led to more restrictive recommendations regarding the use of opioids (Dowell et al., 2016).

Peripheral Opioid Analgesia

With the discovery of opioid receptors on peripheral sensory neurons it became clear that part of the analgesic effect of systemically (e.g., intravenously, subcutaneously, intraperitoneally) applied opioids is mediated outside the CNS in rodent models of inflammatory pain (Labuz et al., 2007; Weibel et al., 2013) and in patients with postoperative pain (Likar et al., 1997; Jagla et al., 2014). It is now generally accepted that opioids are involved in peripheral pain modulation (Stein and Lang, 2009; Stein and Machelska, 2011; Sawynok and Liu, 2014) and considerable effort has been directed into pharmacological approaches focusing on peripheral mechanisms of chronic pain (Gilron and Dickenson, 2014).

Also, neuropathic pain can be alleviated by activation of peripheral opioid receptors (McNicol et al., 2013). This has been shown in several clinical studies (Azad et al., 2000; Ayling et al.,

2014) and in animal models. Regarding the latter, mechanical and heat hypersensitivity following sciatic or spinal nerve ligation were attenuated by several opioids applied to affected paws or at the site of nerve injury in rats (Truong et al., 2003; Kabli and Cahill, 2007; Obara et al., 2009) and mice (Kolesnikov et al., 2007; Hervera et al., 2012; Labuz and Machelska, 2013), while when applied at the site of injury they effectively reduced hypersensitivity to thermal and mechanical stimuli (Labuz and Machelska, 2013).

Additionally, systemic administration of the peripherally acting MOR agonist loperamide effectively reversed the mechanical hyperalgesia after spinal nerve ligation in a rat model of neuropathic pain (Guan et al., 2008; Chung et al., 2012).

Opioid Receptors

Opioid effects are mediated by opioid receptors. They belong to the group of G protein-coupled receptors (GPCRs) and are classified into the three subtypes, δ, κ and μ-opioid receptors (MOR). All GPCRs share a common structure of seven transmembrane domains and their close coupling to G proteins. Opioid receptors couple to heterotrimeric inhibitory $G_{\alpha i/o}$ proteins. Binding of an agonist like morphine induces conformational changes in the opioid receptor leading to intracellular coupling of $G_{\alpha i/o}$ proteins to the C-terminus of the opioid receptor. Dissociation of the G_{α} subunit from the $G_{\beta\gamma}$ subunits of the G protein allows the G_{α} subunit to inhibit adenylate cyclase resulting in reduced production of cyclic adenosine monophosphate (cAMP), whereas the $G_{\beta\gamma}$ subunit directly interacts with ion channels. Specifically, activation of opioid receptors leads to inhibition of calcium channels and TRPV1 channels, and opening of potassium channels, which results in the attenuation of neuronal excitability, and thus analgesia (Stein, 2016).

For a long time, research on analgesic effects of opioids has been exclusively focused on the CNS. Beginning in the early 1980s evidence accumulated suggesting that opioid receptors are not restricted to the CNS. Opioid receptors were also found on primary afferent neurons and cells of the immune system in humans (Stein et al., 1990; Mousa et al., 2007), mice (Manteniotis et al., 2013; Schmidt et al., 2013; Celik et al., 2016) and rats (Stein et al., 2003; Obara et al., 2009; Stein and Machelska, 2011).

In the peripheral sensory neuron, opioid receptors are synthetized in the DRG cell body and transported to central as well as peripheral terminals, where they modulate excitability and transmitter release (Stein et al., 2003; Stein and Machelska, 2011; Vadivelu et al., 2011). Following injury to spinal or sciatic nerve, opioid receptor expression on the mRNA or protein

levels in the DRG was either decreased (Kohno et al., 2005; Obara et al., 2009; Lee et al., 2011), unchanged (Kolesnikov et al., 2007) or elevated (Truong et al., 2003; Kabli and Cahill, 2007). Since activation of peripheral opioid receptors attenuated hypersensitivity in these models (see also above), enhanced function/signaling of opioid receptors may be more predictive for peripheral opioid analgesia in neuropathy than levels of protein expression.

While all three types of opioid receptors might be involved in peripheral analgesic effects, the clinically most relevant type remains the MOR (Stein, 2016). In this project, we thus focused on MOR dependent effects.

1.3.2. Potassium Channels and Neuropathic Pain

Augmented excitability of primary afferents is considered a key feature of neuropathic pain. Since potassium channels have a pivotal role in the regulation of neuronal excitability, AP duration and firing frequency of primary afferents, changes in potassium channel expression or functioning have been implicated as mechanisms of neuropathic pain (Waxman and Zamponi, 2014; Prescott et al., 2014). Therapeutic strategies, however, need to take into account that expression of many of the relevant targets, including potassium channels, is not restricted to the peripheral or central nervous system, but is also relevant in other systems such as heart, endothelium, and kidney (Du and Gamper, 2013; Busserolles et al., 2016). It is therefore important to identify peripheral neuronal mechanisms of neuropathic pain to directly target these in order to avoid peripheral adverse effects such as arrhythmias, in other organ systems.

Hence, identification of potassium channels and their regulators in peripheral sensory neurons presents an opportunity for the development of therapeutic strategies for neuropathic pain.

Classes of Potassium Channels

Potassium channels constitute the largest family of ion channels in mammals, with more than 70 genes coding for them (Waxman and Zamponi, 2014). They are important mediators of cellular excitability in central and peripheral nervous system. These channels allow for potassium ions to selectively cross cellular membranes following the respective electro-chemical gradient. Their gating is regulated by a broad range of intra- and extracellular stimuli, including changes in membrane potential, ion composition, second messenger systems (e.g., IP_3/DAG pathway), and direct binding partners (e.g., G-protein interactions).

Four groups of potassium channels are commonly distinguished by structural and functional criteria: voltage-gated potassium channels (K_v), ion-sensitive potassium channels (K_{Ca}, K_{Na}), two-pore-domain potassium channels (K_{2P}) and inwardly rectifying potassium channels (K_{ir}).

Due to their pivotal role in maintaining the resting membrane potential of neurons, the group of K_{ir} channels has gained particular interest in pain research (Lüscher and Slesinger, 2010; Nagi and Pineyro, 2014).

Inwardly Rectifying Potassium Channels

The term "inwardly rectifying" refers to the generation of large K^+ conductance at potentials negative from equilibrium potential for K^+ (E_K), while these channels permit little current at potentials positive in relation to E_K (see Figure 1.2). Furthermore, at potentials close to resting membrane potential (E_{rest}), they allow a small K^+ outward current, essentially serving as a short circuit for incoming depolarizations (Lüscher and Slesinger, 2010). These features define a key role for K_{ir} in the maintenance of E_{rest}. Therefore, cells expressing high levels of K_{ir} tend to show E_{rest} close to E_K and thus, no spontaneous electrical activity (Hibino et al., 2010).

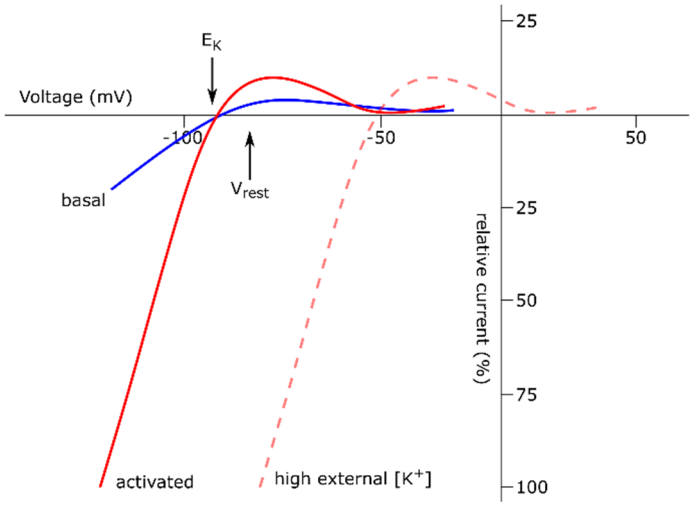

Figure 1.2: Inward rectification of $K_{ir}3$ channels. Current is plotted as a function of voltage. Both basal (blue) and agonist-induced currents (red) show inward rectification, i.e. larger inward current compared to outward current measured at same distance from E_K. Inward rectification becomes more prominent in activated state (red). At potentials close to resting membrane potential (V_{rest}), $K_{ir}3$ channels allow hyperpolarizing outward currents counteracting depolarization and thus stabilizing V_{rest}. High extracellular [K+] shifts the IV-curve towards more positive potentials (dashed red). This is often exploited in patch clamp experiments (see section 2.4.6). (modified after Lüscher et al., 2010)

The inward rectification is the result of an intracellular block by magnesium and polyamines (Matsuda et al., 1987; Lopatin et al., 1994). The activity of K_{ir} channels is regulated by extracellular K^+, membrane bound PIP_2, extracellular H^+ and adenosine triphosphate (ATP) (Whorton and MacKinnon, 2013). Phosphorylation of the channel can, depending on the types of K_{ir}, increase or decrease channel activity (Ippolito et al., 2005).

Another important regulatory mode is protein-protein interaction, which is especially relevant for two subtypes of K_{ir}: ATP-regulated potassium channels (K_{ATP} or $K_{ir}6.x$) and G-protein

coupled inwardly rectifying potassium channels (GIRK or $K_{ir}3.x$). While K_{ATP} is regulated by a colocalized sulfonylurea receptors, $K_{ir}3$ are mainly regulated by $G_{\beta\gamma}$ subunits of G proteins (Figure 1.3). Based on these characteristics, the seven identified families of K_{ir} channels ($K_{ir}1$-7) can be divided into four groups:

i. classical K_{ir} channels ($K_{ir}2.x$)
ii. G protein-gated K_{ir} channels ($K_{ir}3.x$ or GIRK)
iii. ATP-sensitive K_{ir} channels ($K_{ir}6.x$ or K_{ATP})
iv. K^+ transport channels ($K_{ir}1.x$, $K_{ir}4.x$, $K_{ir}5.x$, $K_{ir}7.x$)

(for review see Tsantoulas and McMahon, 2014).

G Protein-Gated Inwardly Rectifying Potassium Channels

Among the group of K_{ir} channels, the family of the G protein-gated K_{ir} channels ($K_{ir}3.1$-4) has gained special interest in pain research. Four channel subunits ($K_{ir}3.1$-4) have been identified, which associate to tetrameric channels with one ion selective pore in their center. Since $K_{ir}3.1$, 3.3 and 3.4 are unable to independently form functional channels, they form heterotetrameric channels, while $K_{ir}3.2$ can form homo- as well as heterotetrameres (Lüscher and Slesinger, 2010). In neuronal tissues, K_{ir} channels are most frequently formed by association of $K_{ir}3.1$, 3.2 and 3.3, with $K_{ir}3.1/3.2$ being the prevailing neuronal channel heterotetramer (Hibino et al., 2010).

$K_{ir}3$ channels form membrane bound signaling complexes with GPCRs such as opioid receptors (Nagi and Pineyro, 2014). Specific N- and C-terminal domains of each channel subunit associate with one $G_{\beta\gamma}$ dimer of the pertussis toxin sensitive heterotrimeric $G_{i/o}$ protein (Whorton and MacKinnon, 2013). Upon binding of the $G_{\beta\gamma}$ subunit to the $K_{ir}3$ monomers, the $K_{ir}3$ channel undergoes conformational changes twisting the pore of the channel open, which allows passage of potassium ions through the channel (Figure 1.3) (Reuveny, 2013). Inwardly rectifying properties of $K_{ir}3$ channels result from a lack of negatively charged residues at the narrow intracellular mouth of the channel as well as intracellular block by magnesium and polyamines (Matsuda et al., 1987; Lopatin et al., 1994; Hilder and Chung, 2013).

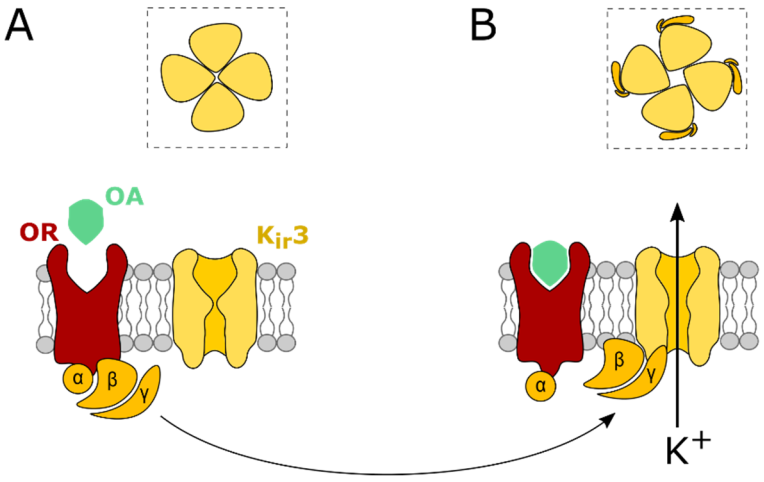

Figure 1.3: Opening of $K_{ir}3$ channels by opioids. (A) Opioid receptors (OR) are coupled to pertussis toxin sensitive heterotrimeric $G_{i/o}$ proteins in close proximity to $K_{ir}3$ channels. In closed state $K_{ir}3$ channel (inset) has low conductance for potassium ions. (B) Binding of opioid receptor agonist (OA) induces conformational changes in opioid receptor leading to dissociation of G protein and subsequent opening of $K_{ir}3$ channel via binding of Gβγ-subunit to $K_{ir}3$ channel subunits. Opening of $K_{ir}3$ channel involves twisting of the pore forming domains (inset).

In the mouse, $K_{ir}3$ channels represent an important postsynaptic effector of GPCRs such as opioid receptors and adrenergic receptors in neurons in the brain and spinal cord, and thus, mediate effects of opioids and α-blockers, respectively (Mitrovic et al., 2003; Lüscher and Slesinger, 2010). $K_{ir}3$ channel involvement in pain modulation was first suggested by research on weaver mice. Weaver mice have a single nucleotide mutation in the pore forming region of K_{ir} 3.2, which leads to reduced activation by GPCRs such as MOR and show loss of opioid induced analgesia (Ikeda et al., 2000). The involvement of $K_{ir}3$ channels in centrally-mediated analgesic effects of opioids was later confirmed in $K_{ir}3.2$ knockout mice (Blednov et al., 2003). In humans, polymorphisms in $K_{ir}3$ genes have been linked to postoperative analgesic opioid requirements and risk for addiction (Nishizawa et al., 2009; Lotsch et al., 2010).

Data on expression and function of $K_{ir}3$ channels in peripheral neurons are scarce.

$K_{ir}3$ mRNA and protein expression and function were shown in rat and human DRG neurons (Gao et al., 2007; Chung et al., 2014; Nockemann et al., 2013; Khodorova et al., 2003; Gorham et al., 2014). After peripheral nerve axotomy $K_{ir}3.1$ / 3.2 subunits were found to be downregulated in rat DRG neurons (Lyu et al., 2015).

In contrast, the data on $K_{ir}3$ in the mouse are conflicting. Two studies did not find $K_{ir}3$ mRNA or protein in DRGs or skin (Nockemann et al., 2013; Mitrovic et al., 2003), while others detected $K_{ir}3$ mRNA in DRGs of naïve wildtype mice (Manteniotis et al., 2013; Saloman et al., 2016).

Functional coupling of opioid receptors and $K_{ir}3$ channels has been reported and linked to peripheral opioid analgesia in a model of inflammatory pain in mice (Nockemann et al., 2013). So far, there is no data on functional coupling of opioid receptors and $K_{ir}3$ channels in peripheral sensory neurons under neuropathic pain conditions.

1.4. Opioids and Potassium Channels in Peripheral Pain Control

Whereas regulation of calcium channels represents the best known mechanism of opioid analgesia, potassium channels have gained increased attention in the pain field as potential targets for novel analgesics due to their crucial role in the regulation of neuronal excitability (Ocaña et al., 2004; Tsantoulas and McMahon, 2014).

Importantly, potassium channels have been shown to be relevant components of peripheral opioid analgesia.

Several members of the K_{2P} family (TREK, TESK) have been detected in primary afferent neurons (Han et al., 2016; Tulleuda et al., 2011) and were modulated by opioids (Cho et al., 2016). Peripheral opioid analgesia in rats has been linked to $K_{ir}3$ channel (Chung et al., 2014). However, in wild-type mice, application of opioids into inflamed peripheral tissue produced very weak or no analgesic effect. Interestingly, in knock-in mice selectively expressing $K_{ir}3$ channels in peripheral sensory neurons, MOR agonist DAMGO induced analgesia comparable to that seen in rats. Electrophysiological experiments further confirmed functional coupling of MOR to $K_{ir}3$ channels in DRG neurons in rats and $K_{ir}3$ knock-in mice. These data suggested that $K_{ir}3$ is necessary and sufficient for peripheral opioid-mediated analgesia (Nockemann et al., 2013).

Interestingly, however, in neuropathic pain models, opioids were found to alleviate hypersensitivity via activation of peripheral MORs in wild-type mice (Kolesnikov et al., 2007; Hervera et al., 2012; Labuz and Machelska, 2013) (see also section 1.3.1). Therefore, these findings raise the question whether $K_{ir}3$ channels are expressed and functionally coupled to MOR in peripheral sensory neurons in mice following neuropathy. So far, this question has not been addressed in literature and has thus been the main focus of this project.

1.5. Objectives and Hypothesis

The main goal of this thesis was:

To investigate changes in opioid induced potassium conductance upon peripheral nerve injury in mice.

The central hypothesis of this work was as follows:

A lesion to a peripheral nerve in the mouse results in enhanced opioid-induced potassium conductance in the corresponding sensory neurons.

As a model of neuropathic pain, a chronic constriction injury (CCI) of the sciatic nerve was used. This model resembles human neuropathy resulting from trauma to peripheral nerves with some functional preservation of the innervation (e.g., nerve entrapment or compression) (Bennett and Xie, 1988). To examine effects of CCI on DAMGO induced changes in potassium channel conductance, potassium imaging and patch clamp were employed. Recordings were carried out in cultured sensory neurons from DRGs of naïve mice and mice exposed to CCI. As a positive control, equivalent experiments were performed in transfected human embryonic kidney (HEK) 293 cells.

2. Materials & Methods

2.1. Animals

Wild-type male C57BL/6J mice (18-35 g, 6-8 weeks old; Janvier Laboratories, France) were housed in groups of 2 to 4 per cage lined with ground corncob bedding. Standard laboratory rodent chow and tap water were available ad libitum. Rooms were maintained at 22°C and at a relative humidity between 40% and 60%. A 12 h / 12 h (8 am/8 pm) light/dark cycle was used. Experiments were approved by the State animal care committee (Landesamt für Gesundheit und Soziales, Berlin, Germany; protocols T0132/07 and G0277/13) and strictly followed the guidelines of the International Association for the Study of Pain (IASP) (Zimmermann, 1983) and the ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Materials

Cell Line	
HEK 293 cells	German collection of microorganisms and cell cultures (DSMZ), Braunschweig, Germany
Antibiotics	
Penicillin/streptomycin	Biochrom Merck, Berlin, Germany
Plasmids	
pFLAG-CMV-K_{ir}3.2	kindly provided by Dr. Dinah Nockemann
pcDNA-3.1-MOR	kindly provided by Prof. Dr. Christian Zöllner
Enzymes	
Collagenase type I	Sigma-Aldrich Chemie, Munich, Germany
Trypsin type IX-S	Sigma-Aldrich Chemie, Munich, Germany

Pharmacological compounds

Barium chloride	Sigma-Aldrich Chemie, Munich, Germany
DAMGO	Sigma-Aldrich Chemie, Munich, Germany
Glibenclamide	Sigma-Aldrich Chemie, Munich, Germany
Isoflurane	AbbVie, Ludwigshafen, Germany
Tetraethylammonium chloride (TEA)	Sigma-Aldrich Chemie, Munich, Germany

Media and Sera

Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich Chemie, Munich, Germany
DMEM/Ham's F12	Biochrom Merck, Berlin, Germany
Horse serum	Biochrom Merck, Berlin, Germany

Chemicals and Reagents

Calcium chloride ($CaCl_2$)	Sigma-Aldrich Chemie, Munich, Germany
Deionized water	B. Braun Melsungen, Melsungen, Germany
D-Glucose	Sigma-Aldrich Chemie, Munich, Germany
Ethanol 99.9%	B. Braun Melsungen, Melsungen, Germany
Ethylene glycol-tetraacetic acid (EGTA)	Sigma-Aldrich Chemie, Munich, Germany
Hydroxyethyl-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie, Munich, Germany
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich Chemie, Munich, Germany
Magnesium-ATP (MgATP)	Sigma-Aldrich Chemie, Munich, Germany
PBS w/o Ca/Mg	Biochrom Merck, Berlin, Germany
Poly-L-lysine	Sigma-Aldrich Chemie, Munich, Germany
Potassium chloride (KCl)	Merck, Berlin, Germany
Potassium hydroxide (KOH)	Sigma-Aldrich Chemie, Munich, Germany
Sodium chloride (NaCl)	Merck, Berlin, Germany

Sodium hydroxide (NaOH)	Sigma-Aldrich Chemie, Munich, Germany
Sodium-ATP (NaGTP)	Sigma-Aldrich Chemie, Munich, Germany
Trypan blue 0.5 %	Biochrom Merck, Berlin, Germany
X-tremeGENE HP DNA tansfection reagent	Sigma-Aldrich Chemie, Munich, Germany

Kits

FluxOR™ potassium ion channel assay	ThermoFisher Scientific, USA

Consumables

Borosilicate glass capillaries GB150F8P	Science Products, Hofheim, Germany
Cell strainers 40 µm pores	BD Biosciences, Bedford, USA
Culture dishes Ø 35 mm	Techno Plastic Products, Switzerland
Eppendorf tubes 2 ml	Eppendorf, Hamburg, Germany
Falcon cell culture flasks	BD Biosciences, Bedford, USA
Falcon tubes 50 ml	BD Biosciences, Bedford, USA
Glass cover slips Ø 24 mm	VWR International, Darmstadt, Germany
Silk sutures (4-0)	Ethicon Deutschland, Norderstedt, Germany
Silver wire Ø 0.25 mm	Science Products, Hofheim, Germany
Syringe filters 0.2 µm	BD Biosciences, Bedford, USA

Other Supplies

Micro spring scissors	Fine Science Tools, Heidelberg, Germany
Surgical scissors	Fine Science Tools, Heidelberg, Germany
Fine forceps	Fine Science Tools, Heidelberg, Germany
Surgical forceps	Fine Science Tools, Heidelberg, Germany

Equipment

Amplifier EPC-10	HEKA Elektronik, Lambrecht, Germany
Camera CCD IMAGO	TILL Photonics, Munich, Gerrmany
Camera digital sight DS-U3 / DS-QiMc	Nikon Instruments Europe
CO_2 incubator	Heraeus Holding, Hanau, Germany
Computer Optiplex GX280	Dell, Frankfurt a. M., Germany
HS18 laminar air flow	Heraeus Holding, Hanau, Germany
Micromanipulator InjectMan	Eppendorf, Hamburg, Germany
Micropipette puller P-97	Sutter Instrument, Novato, USA
Microscope Axiovert	Carl Zeiss Microscopy, Oberkochen, Germany
Microscope Eclipse TE2000-S	Nikon Instruments Europe
Minispin table top centrifuge	Eppendorf, Hamburg, Germany
Multifuge 4KR centrifuge	Heraeus Holding, Hanau, Germany
Objective Fluar 10x/0.50	Carl Zeiss Microscopy, Oberkochen, Germany
Objective PlanFluor 20x / 0.50	Nikon Instruments Europe
Perfusion pressure kit VPP-6	Warner Instruments, Hamden, USA
pH-Meter MP220	Mettler Toledo
Polychrome V monochromator	TILL Photonics, Munich, Gerrmany
Pump IPC	Ismatec Cole-Parmer, Wertheim, Germany
Six channel valve controller VC-6	Warner Instruments, Hamden, USA
Soldering iron	Toolcraft Conrad Electronic SE, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Ultrapure water systems (Direct-QTM 5)	Millipore, Merck, Darmstadt, Germany
Vortexmixer (2TM Mixer 7-2020)	Neolab, Heidelberg, Germany
YFP filter 535±15 nm	Nikon Instruments Europe

Software

Citavi v5.4	Swiss Academic Software, Switzerland
Nest-o-Patch v1.2	Viatcheslav Nesterov, GNU General Public License. https://nestopatch.sourceforge.io
NIS Elements v4.30	Nikon Instruments Europe
Office 2013	Microsoft Corporation, Redmond, USA
Prism v6	GraphPad Software, La Jolla, USA
Pulse v8.8	HEKA Elektronik, Lambrecht, Germany
TILL Vision v4.01	TILL Photonics, Munich, Gerrmany

Buffers Used in Potassium Imaging Experiments

Loading buffer	Deionized water, PowerLoad™ concentrate, FluxOR™ reagent reconstituted in DMSO, FluxOR™ assay buffer, probenecid reconstituted in deionized water
Assay buffer without channel blockers	Deionized water, FluxOR™ assay buffer, probenecid reconstituted in deionized water
Assay buffer with channel blockers	Deionized water, FluxOR™ assay buffer, probenecid reconstituted in deionized water, 20 mM TEA, 20 µM glibenclamide
Stimulus buffer, vehicle	Deionized water, FluxOR™ chloride-free buffer, Tl_2SO_4 concentrate
Stimulus buffer, DAMGO	Deionized water, FluxOR™ chloride-free buffer, Tl_2SO_4 concentrate, 1-100 µM DAMGO

Buffers Used in Patch Clamp Experiments

Extracellular low potassium	140 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, 2.6 mM D-glucose, adjusted to pH 7.4 using NaOH
Extracellular high potassium, pure	140 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, adjusted to pH 7.4 using KOH
Extracellular high potassium, DAMGO	140 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, 10 µM DAMGO, adjusted to pH 7.4 using KOH
Extracellular high potassium, $BaCl_2$	140 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, 3 mM $BaCl_2$, adjusted to pH 7.4 using KOH
Intracellular	5 mM NaCl, 122 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 11 mM EGTA, 4 mM MgATP, 0.25 mM NaGTP, adjusted to pH 7.4 using KOH

2.3. Characteristics of Pharmacological Agents and Assay Components

DAMGO is a synthetic opioid peptide acting as a selective MOR agonist (Handa et al., 1981). It was used to activate MOR in potassium imaging and patch clamp experiments.

Thallium is a metal showing similarities to some alkali metals, especially potassium, with which it shares a very similar atomic radius. It is well known to permeate potassium channels quite freely (Hille, 1973) and was thus used as a surrogate for potassium in the FluxOR Assay (Geng et al., 2009; Weaver et al., 2004).

Barium is known to block a variety of potassium channels, including inwardly rectifying potassium channels such as $K_{ir}3.x$ or K_{ATP} (Alagem et al., 2001) and was thus used to block DAMGO induced currents in patch clamp experiments.

TEA is a commonly used blocker of K_v channels (Hille, 1967) and was used to unmask possible K_{ir} currents in potassium imaging experiments.

Glibenclamide belongs to the class of sulfonylureas and is known to block K_{ATP} channels by binding to their regulatory subunit SUR1 (Kawano et al., 2009) and was used to unmask possible $K_{ir}3$ currents in potassium imaging experiments.

2.4. Methods

2.4.1. Chronic Constriction Injury

The Chronic Constriction Injury (CCI) of the sciatic nerve, first described by Bennett and Xie (1988), has been used as a model to study mononeuropathies for more than two decades. It resembles clinical traumatic nerve damage such as entrapment or compression, and reliably results in behavior indicating mechanical and thermal hyperalgesia and allodynia as well as spontaneous pain in rodents (Labuz and Machelska, 2013).

To induce CCI, mice were deeply anesthetized with isoflurane, the sciatic nerve was exposed at the level of the right mid-thigh, and three loose silk ligatures (4-0) were placed around the nerve with about 1-mm spacing (Figure 2.1). The ligatures were tied until they elicited a brief twitch in the respective hind limb. The wound was closed with silk sutures (Labuz et al., 2009; Labuz and Machelska, 2013). This procedure was performed by a member of our group, Dr. Özgür Celik.

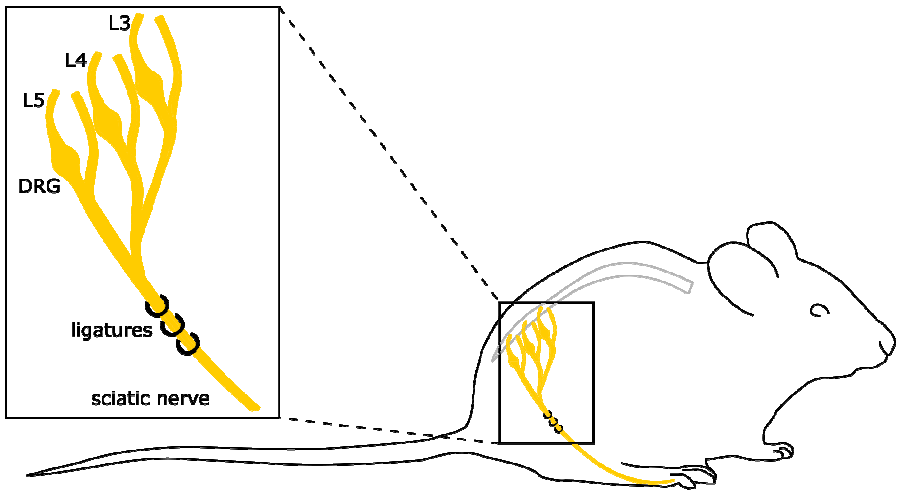

Figure 2.1: Chronic constriction injury model. Scheme illustrating the ligation of the sciatic nerve in relation to the respective nerve roots and lumbar (L) DRGs in the mouse.

2.4.2. DRG Tissue Preparation and Neuron Culture

DRGs were isolated from naïve mice and from mice two days after CCI induction. Mice were killed by isoflurane overdose, the vertebral column was removed, washed in PBS and placed in

ice-cold PBS for dissection of the DRGs. After laminectomy, the lumbar (L) DRGs innervating sciatic nerve (L3-5) were identified and dissected. From animals subjected to CCI, DRGs ipsilateral and contralateral to the CCI were extracted and further handled in separate cultures. From naïve animals, DRGs were extracted from the right side only. DRGs were collected in ice-cold serum-free working medium (DMEM/HAM's F12 supplemented with 1% penicillin/streptomycin). For potassium imaging experiments, DRGs from 2 – 4 animals were pooled to obtain 12 samples for 3 – 4 technical replicates per treatment and experiment. For patch clamp experiments, DRGs from one animal were sufficient to obtain 10 samples for up to 10 technical replicates per experiment. All further handling of the tissue was performed under a laminar air flow hood under sterile conditions. The collected tissue was then incubated in 1.25% collagenase for 50 min at 37°C in a thermoshaker, washed with PBS and incubated in 2.5% trypsin for 5 min at 37°C in a thermoshaker. After digestion, the tissue was triturated using plastic pipette tips and subsequently filtered through a 40-µm cell strainer. The filtrate was centrifuged, the supernatant discarded and the cell pellet resuspended in 300-1000 µl culture medium (DMEM/HAM's F12 supplemented with 1% penicillin/streptomycin and 10% horse serum), depending on the required cell density. The cell suspensions (30–100 µl) were then seeded onto poly-L-lysine coated glass cover slips (24 mm) or plastic culture dishes (35 mm) and kept in an incubator for 1h to allow the cells to settle down. After that, the cell cultures were topped up to a total of 2 ml of culture medium and kept in an incubator at 37°C with 5% CO_2 for 20–30 h. Cell viability was evaluated before first experiments by Trypan Blue exclusion assay.

2.4.3. HEK 293 Cell Culture

Originally derived from human embryonic kidney cells, HEK 293 is a cell line now widely used in biomedical research. HEK 293 cells were maintained in Dulbeco's modified eagle medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum in an incubator at 37°C with 5% CO_2. Cells were split every 2-3 days.

2.4.4. Transfection of HEK 293 Cells

Transfection is a technique used to introduce DNA of interest into eukaryotic cells by non-viral methods. HEK 293 cells transfected with MOR and $K_{ir}3.2$ were used as a positive control in potassium imaging and patch clamp experiments.

HEK 293 cells were seeded onto glass cover slips (24 mm) or plastic culture dishes (35 mm) one day prior to transfection. The transfection mixture was composed of 1 µg MOR DNA, 1 µg $K_{ir}3.2$ DNA, 6 µl XtremeGene added to 88 µl pure Dulbeco's modified eagle medium per cover slip or culture dish. After 15 min of incubation at room temperature, the transfection mixture was added to the HEK 293 cell cultures. Transfected cultures were kept in an incubator at 37°C with 5% CO_2 for 48 h before starting experiments.

2.4.5. Potassium Imaging

A real-time imaging approach was employed to investigate MOR agonist (DAMGO) dependent changes in potassium permeability in mouse DRG neurons and HEK 293 cells, using the FluxOR Potassium Imaging Assay (Invitrogen Molecular Probes, 2009). This assay relies on shared properties of potassium and thallium ions, i.e. the permeability of potassium channels to thallium. Cells are loaded with a non-fluorescent ester form of the thallium-sensitive dye, which is then intracellularly cleaved into the de-esterified fluorogenic thallium-sensitive form. The loading buffer is then washed off the cells and replaced by a dye-free assay buffer. When a stimulus buffer containing thallium is added to the cells, which leads to opening of potassium channels, the thallium follows its concentration gradient into the cells activating the dye. Changes in fluorescence intensity can then be monitored in real time as an indirect measure of potassium channel activity (Figure 2.2).

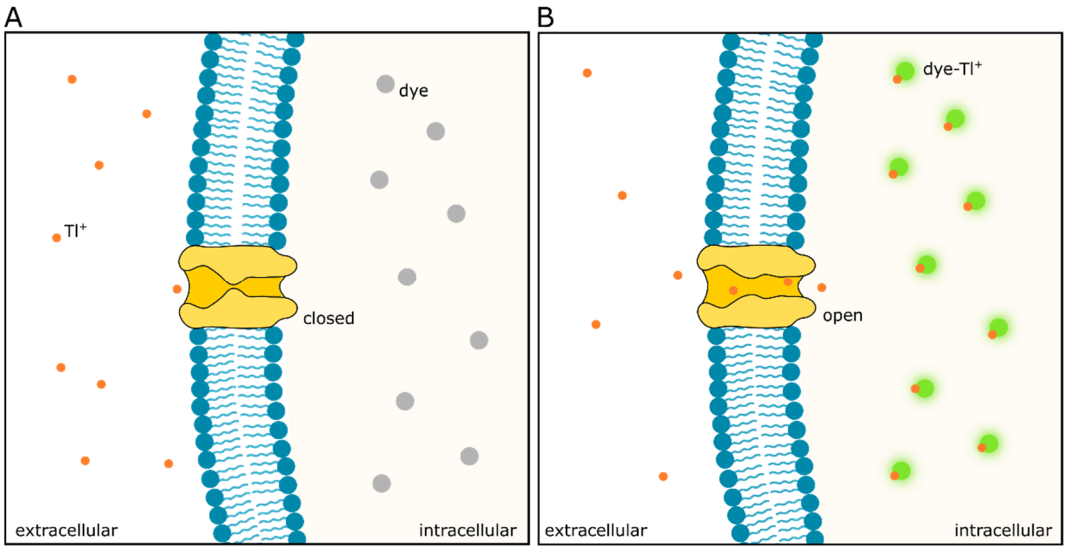

Figure 2.2: Assay principle of the FluxOR potassium imaging assay. Cells are loaded with the thallium (Tl^+) sensitive FluxOR dye. Basal fluorescence of cells loaded with the FluxOR dye is low while the dye is not in contact with thallium (A). Once thallium enters the cells through potassium channels, it binds to the dye, thereby activating it (B). This can be monitored in real time as increase in specific fluorescence (Modified after invitrogen™ Molecular Probes® 2009).

Originally, the assay was designed as a tool for high throughput screening in cell lines using a microplate reader (Beacham et al., 2010; Titus et al., 2009). Since we intended to investigate DRG mouse neurons, the assay was adopted for use in primary neuronal cultures using fluorescence microscopy, as described earlier (Nockemann et al., 2013).

For imaging experiments, DRG neurons from naïve mice, and neurons from ipsi- and contralateral DRGs of mice exposed to CCI (see section 2.4.1), were used 20–30 h after preparation of the cultures. For experiments in HEK 293 cells transfected with MOR and $K_{ir}3.2$, the cells were used 2 days after transfection.

For real time imaging of thallium uptake in HEK 293 cells and DRG neurons, culture medium was removed from the cells, followed by washing with PBS and addition of loading buffer. After incubation for 90 min at room temperature in the dark, cells were washed again with PBS. Imaging assay buffer was added and cells were incubated for 30 min at room temperature in the dark. The glass cover slip was then transferred to the microscope (Nikon eclipse TE2000-S) and a cell rich area was selected for recording. For experiments with blockers of K_v and K_{ATP} channels, TEA (20 mM) and glibenclamide (20 µM) were added to the assay buffer. Separate experiments were performed omitting the potassium channel blockers.

Time series of fluorescent images were acquired with one picture being taken every second with an exposure time of 100 ms (using TILL IMAGO CCD or Digital Sight DS-U3/DS-QiMc cameras). The dye was excited using a wavelength of 488 nm (using a TILL Photonics Polychrome V monochromator) and emission was quantified at 525 nm. After 20 s of baseline recording, DAMGO (1 µM, 10 µM, 100 µM final concentration in recording chamber) or vehicle buffer (see section 2.2) were added to the recording chamber and subsequent change in fluorescence was recorded for 3 min. Concentrations of DAMGO were based on previous studies (Nockemann et al., 2013) and preliminary experiments performed in our lab.

Images from experiments in mouse DRG neurons were analyzed using TillVision v4.01 software. After background subtraction, regions of interest (ROI) were chosen manually. Selection of cells for analysis was guided by their morphology and size: Medium size (diameter 20–35 µm) round cells were chosen for recording and analysis, whereas small size (diameter < 20 µm) round or spindle shaped cells, assumed to be glial cells or fibroblasts (Liu et al., 2013; Malin et al., 2007), were neglected. Mean fluorescence intensity was then calculated for each ROI at each time point.

Images from experiments in HEK 293 cells were analyzed using NIS Elements v4.30 software. Since HEK 293 cells are homogenous, all cells showing typical, undamaged morphology were analyzed. After background subtraction, ROI were chosen manually, and mean fluorescence intensity was calculated for each ROI at each time point.

2.4.6. Patch Clamp

Since its invention in the late 1970s (Neher and Sakmann, 1976) and refinement in the early 1980s (Hamill et al., 1981; Sakmann and Neher, 1984), the patch clamp method has enabled scientists to study the bioelectrical properties of cell membranes, including their ion channels, in unprecedented details. Patch clamp is now a common tool in many areas of biomedical research, particularly in fields focused on neuronal cells.

In my experiments, the patch clamp technique was used in whole-cell mode. In this mode, a fine glass pipette, filled with an electrolyte solution resembling the cell's intracellular solution (see section 2.2), is brought into close contact with the cell membrane to form a tight seal between lipid bilayer and glass surface. Using a short but strong suction pulse, the membrane patch under the pipette tip is then ruptured, which allows access to the cytoplasm. Currents between the electrode placed in the pipette's solution and the reference electrode placed in the bath solution surrounding the cell can then be amplified and measured, reflecting ion flux over the cell membrane (Figure 2.3).

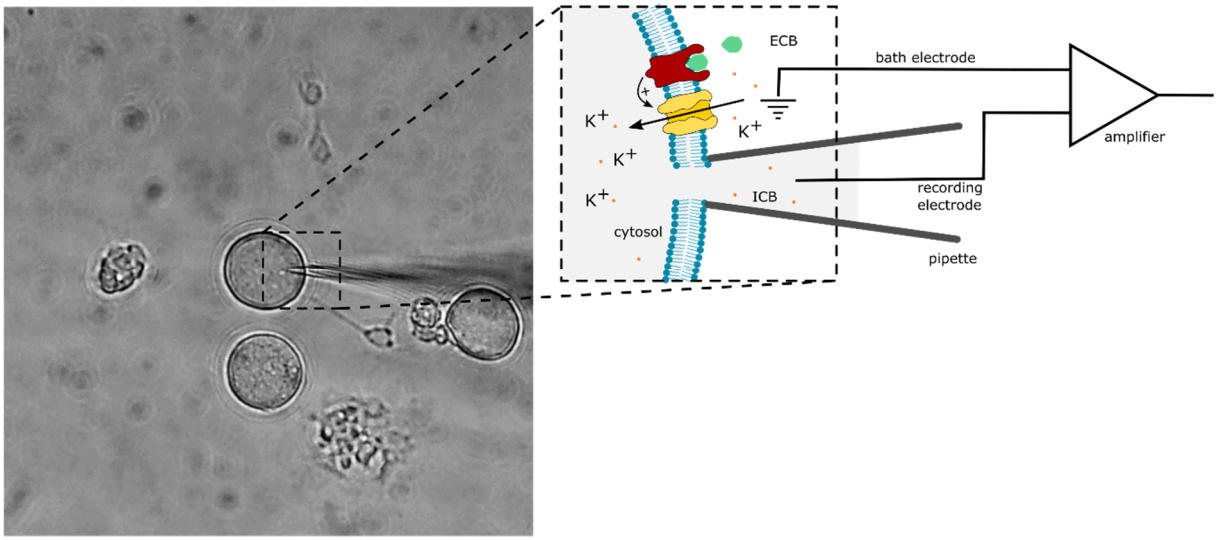

Figure 2.3: Whole-cell patch clamp setup. Left: Bright field microscopy image of dissociated mouse DRG neurons with patch pipette in patching position. Right: Simplified scheme illustrating the setup to perform patch clamp recordings in whole-cell mode. A fine glass pipette containing intracellular buffer (ICB) and a recording electrode connected to the amplifying probe is brought in close contact with the cell membrane allowing it to form a tight seal with the pipette tip. Using a short strong suction pulse, the membrane patch at the pipette tip is ruptured giving access to the intracellular space. Whole-cell currents over the cell membrane can then be measured between recording electrode in contact with ICB and reference electrode in extracellular buffer (ECB). The scheme illustrates recording of opioid mediated potassium (K^+) currents through $K_{ir}3$ channels.

For recordings, DRG cells of naïve mice and neurons from ipsilateral DRGs of mice exposed to CCI, were used 20–30 h after cultivation. Cells with medium diameter of 20–35 µm were selected for recording, similar to potassium imaging experiments (see section 2.4.5) (Stucky and Lewin, 1999). HEK 293 cells were recorded 40 – 50 h after plating (untransfected cells) or transfection with MOR and $K_{ir}3.2$.

After washing with PBS, cells were bathed in low potassium extracellular solution, visualized using a Zeiss Axiovert 200 inverse microscope. Patch pipettes (resistance 3.5–8 MΩ) were fabricated from Borosilicate capillaries glass with filament using a Sutter P-97 puller (Sutter Instrument Co, USA). Currents were amplified and recorded using an EPC-10 patch amplifier and Pulse software (HEKA, Germany), and were sampled at a frequency of 100 Hz.

Cells were superfused by steady flow of buffer at a flow rate of approximately 800–1000 µl/min using a pressurized application system (Perfusion Pressure Kit VPP-6, Warner Instruments, USA) and a suction pump. Test compounds, DAMGO (10 µM) and $BaCl_2$ (3 mM) were applied using a perfusion valve control systems (VC-6, Warner Instruments, USA) to switch between vehicle buffer and buffers containing the test compounds. All recordings were performed at room temperature. Fast capacitive currents (i.e., pipette potential) were cancelled before seal formation. After reaching "giga-seal", the membrane patch was ruptured to achieve whole-cell configuration.

In DRG neurons, resting membrane potential was estimated in current-clamp mode shortly after gaining whole-cell access and APs were recorded in current-clamp mode using stepwise increasing current injections of 100 ms duration from 100 to 600 pA. Only cells showing proper AP overshoot were included for further experiments. Cell capacitance, series and input resistance were monitored by applying test pulses of 10 mV for 10 ms before each recording.

DAMGO-induced currents were recorded in voltage-clamp mode at a constant holding potential of -80 mV in high K^+ buffer for 120 s (Nockemann et al., 2013). Hyperpolarized state and high concentration of K^+ in extracellular buffer were used to increase the electro-chemical gradient for K^+ to drive it into the cell when inwardly rectifying potassium channels are opened (see Figure 1.2). To reduce "stress" on the cells, high K^+ buffer was washed in carefully over a period of 2 min and cells were allowed to stabilize for at least 2 min before recording.

The analysis of patch-clamp recordings was performed using Nest-o-Patch v1.2 and Prism v6 software. (GraphPad Software, Inc). Effects of DAMGO were measured as departure from holding current while running vehicle buffer. Cells were considered responders to DAMGO application if the resulting current was larger than three times the noise range. Effects of $BaCl_2$ were measured as departure from holding current while running DAMGO buffer. Drift of baseline was corrected using the Nest-o-Patch baseline correction tool when necessary.

2.4.7. Statistical Analyses

Potassium Imaging

Data analysis was performed using Prism v6 (GraphPad Software, Inc). Mean fluorescence intensity of the first 20 time-points was used as baseline fluorescence (F_0) of each ROI. Change in fluorescence (ΔF) relative to baseline was then calculated for each time point ($\Delta F = F/F_0$). To compare ROIs, area under the curve (AUC) of each ROI was used as a measure of cumulative change in fluorescence for each cell.

For vehicle treatment, each DAMGO dose and condition (DRG from naïve mice, ipsi- and contralateral DRG from CCI mice, HEK 293 cells), all data points are shown. Data points from experiments in DRG cells represent single cell values to reflect heterogeneity of cell types in DRGs. Exact cell numbers per group are stated in the figure legends (see sections 3.1.1 and 3.1.2). Data points from experiments in HEK 293 cells represent technical replicates, where one data point represents one plate of cells recorded at the same time, to reflect the homogeneity

of the cell population consisting of HEK 293 cell clones. Numbers of plates per group are stated in the figure legends (see section 3.1.3). The data are expressed as dots representing AUC of ΔF for single cells or plates with bars representing means, as specified in each figure legend. Single cell kinetics is shown as ΔF over time in exemplary traces.

Statistical analysis was performed based on single cell values for experiments conducted on mouse neurons, and based on technical replicates for experiments performed on HEK 293 cells.

The data distribution was assessed using the D'Agostino-Pearson test and the following statistical tests were chosen accordingly. Hence, Kruskal-Wallis one-way analyses of variance (ANOVA) followed by Dunn's post-hoc test were used to analyze (i) effects of different doses of DAMGO compared to vehicle treatment for each condition in mice (naïve, CCI ipsilateral, CCI contralateral), and (ii) effects of CCI ipsilateral groups compared to naïve as well as CCI contralateral groups for each dose of DAMGO and vehicle treatment, respectively. The effects of DAMGO compared to vehicle treatment in transfected HEK 293 cells were analyzed using an unpaired t-test. Differences were defined as statistically significant if $p < 0.05$.

Patch Clamp

Data are shown as individual data points representing single cell currents with bars indicating medians. The number of cells per group was 13 – 33; the exact numbers are given in figure legends (see section 3.2). The data distribution was assessed using the D'Agostino-Pearson test and the following statistical tests were chosen accordingly. Thus, the effect of CCI on DAMGO-induced currents was compared to DAMGO-induced currents in naïve mice using Mann-Whitney U test. The same test was used to compare DAMGO-induced currents between transfected and untransfected HEK 293 cells. These analyses were performed for all recorded cells, including DAMGO-responders and non-responders. Reversibility of DAMGO-induced currents by $BaCl_2$ was assessed by pairwise comparison of absolute values of respective currents using a paired t-test.

To compare ratios of DAMGO-responders to DAMGO-non-responders between naïve and CCI mice as well as between untransfected and transfected HEK 293 cells, the Fisher's exact test was used. This test was employed since subgroup analysis of responders did not reach minimum number of values to apply statistical tests on continuous outcomes. The differences were defined as statistically significant if $p < 0.05$.

3. Results

3.1. Real Time Fluorescence Microscopy to Investigate Effects of MOR Agonist DAMGO on Potassium Channel Conductance in Mouse DRG Neurons and HEK 293 cells

To investigate the effects of DAMGO on potassium flux in mouse DRG neurons and in HEK 293 cells, a real-time fluorescence imaging approach using the FluxOR assay was employed. This assay makes use of a thallium-sensitive dye, which allows to study the thallium flux through the cell membrane. Hence, in this assay, increase in fluorescence representing cellular thallium uptake serves as a surrogate measure of potassium flux into the cells.

3.1.1. No Change in $K_{ir}3$ Channel Conductance upon DAMGO Application in DRG Neurons of Naïve and CCI Mice

First experiments were performed in cultured DRG neurons isolated from naïve mice and mice exposed to nerve injury (2 days following CCI). Potassium channel blockers TEA (20 mM) and glibenclamide (20 µM) were used to block K_v or K_{ATP} channels, respectively, in order to investigate possible effects mediated by $K_{ir}3$ channels. A DAMGO dose of 10 µM is the commonly used dose to investigate MOR effects in imaging and electrophysiology experiments (Nockemann et al., 2013). Additionally, a 100 µM dose was used since no effects of 10 µM were seen.

Application of stimulus buffer containing DAMGO and thallium after 20 s of base-line recording resulted in increased fluorescence, representing thallium uptake, in all DRG cells tested in all experiments (see Figure 3.1 for exemplary trace).

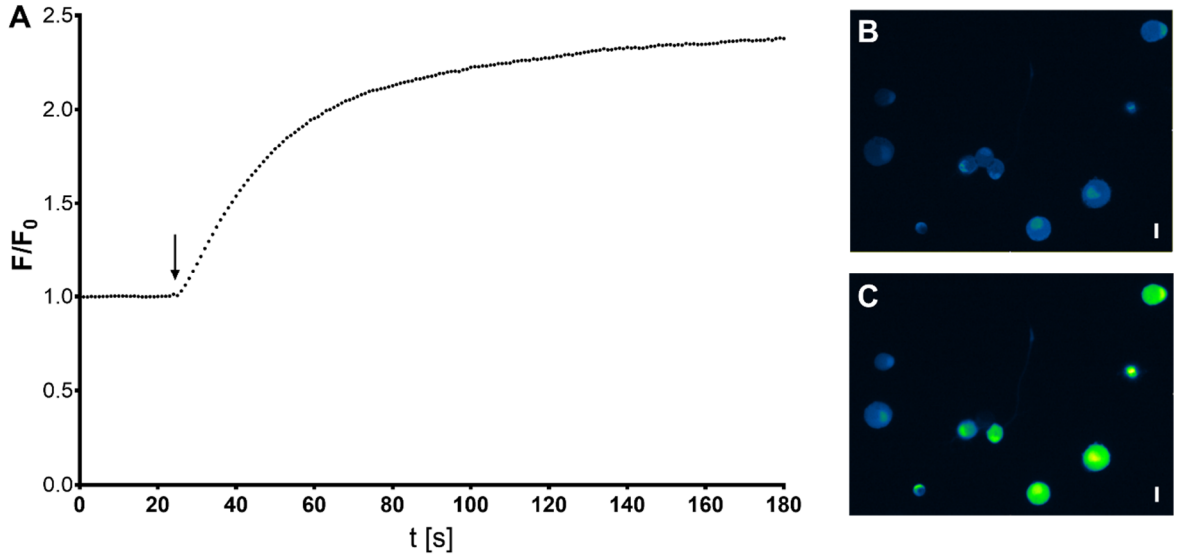

Figure 3.1: Real-time imaging of thallium flux in isolated mouse DRG cells. (A) Exemplary trace of thallium uptake in one isolated naive mouse DRG cell. Cells were loaded with the thallium-sensitive FluxOR dye. Fluorescence (approximate excitation and emission: 488/525 nm), indicating thallium uptake, increased upon addition of stimulus buffer containing thallium and DAMGO (10 µM). Assay buffer contained the potassium channel blockers TEA and glibenclamide. Arrow indicates application of the buffer. (B, C) Exemplary fluorescence microscopy images showing DRG cells (B) before, t = 20 s, and (C) after, t = 180 s, application of stimulus buffer. Scale bars: 20 µm.

However, there were no significant differences in thallium uptake between DAMGO (10 µM and 100 µM) and vehicle treatment in cells isolated from DRGs of naïve mice or DRGs ipsilateral and contralateral to CCI. There were also no significant differences between DRG cells of naïve mice, DRG ipsilateral and contralateral to CCI in control, DAMGO (10 µM) and DAMGO (100 µM) groups (Figure 3.2).

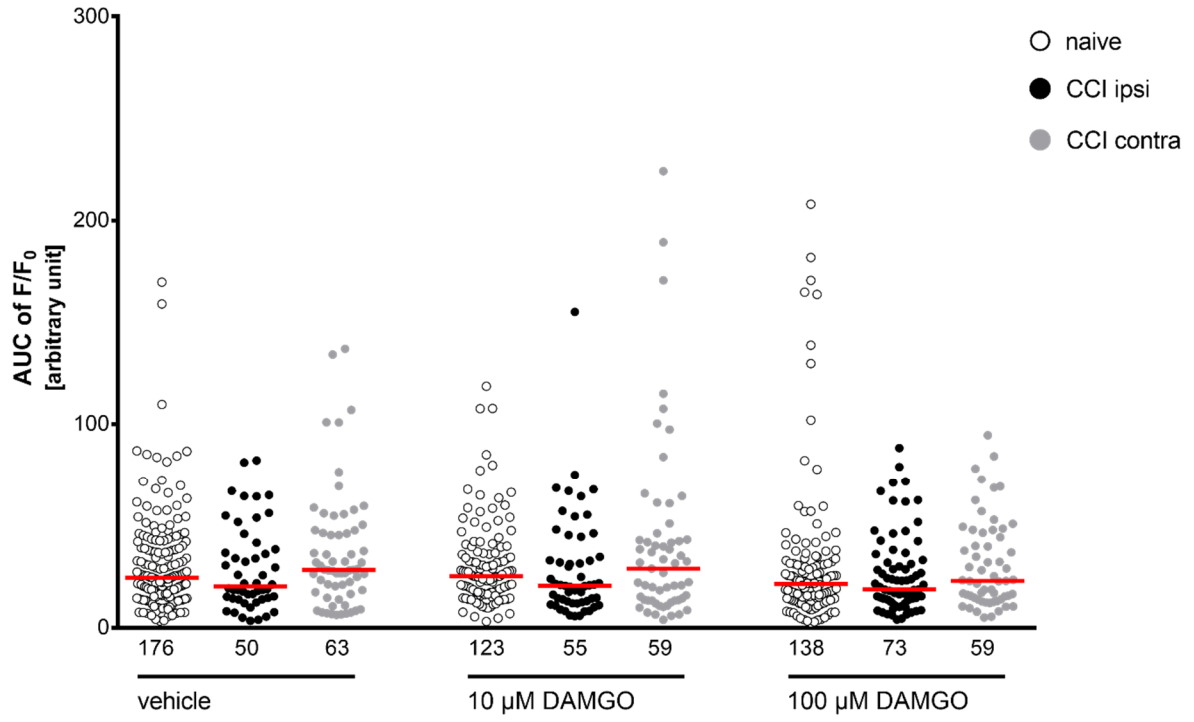

Figure 3.2: No effects of DAMGO on thallium uptake in mouse DRG neurons in the presence of K_v and K_{ATP} channel blockers, measured as increase in fluorescence intensity of the FluxOR dye. Data are quantified as area under the curve (AUC) of change in fluorescence intensity (F/F_0) after application of stimulus buffer containing thallium and DAMGO (10-100 µM as indicated) or thallium alone (vehicle). The assay buffer also contained blockers of K_v (TEA) and K_{ATP} (glibenclamide). Single dots represent single cell values, bars indicate medians. Numbers below indicate number of cells from at least 3 cultures per group, from 4 animals per culture (p=0.0641; Kruskal-Wallis one-way ANOVA).

3.1.2. No Enhancement of Potassium Channel Conductance upon DAMGO Application in DRG Neurons of Naïve and CCI Mice

In order to investigate possible effects of CCI on potassium channels other than K_{ir}, blockers of K_v or K_{ATP} channels (TEA and glibenclamide) were omitted in the subsequent experiments. In addition to the standard dose of 10 µM DAMGO, in these experiments also 1 µM and 100 µM were used to establish a dose-response relationship.

In these experiments, DAMGO (1 µM and 10 µM) did not result in significantly increased thallium uptake compared to vehicle in cells from DRGs of naïve mice or DRGs ipsilateral and contralateral to CCI (Figure 3.3). In contrast, DAMGO (100 µM) significantly reduced thallium uptake compared to vehicle in DRG cells from naïve mice and cells from DRGs ipsilateral to CCI, but not contralateral to CCI (Figure 3.3).

Additionally, in vehicle-treated cells, there was a significantly lower thallium uptake in cells from DRGs contralateral to CCI compared to ipsilateral DRGs and to DRGs of naïve mice. For

DRGs contralateral to CCI, the effect in the vehicle-treated group was significantly lower compared to DAMGO (1 µM) (Figure 3.3).

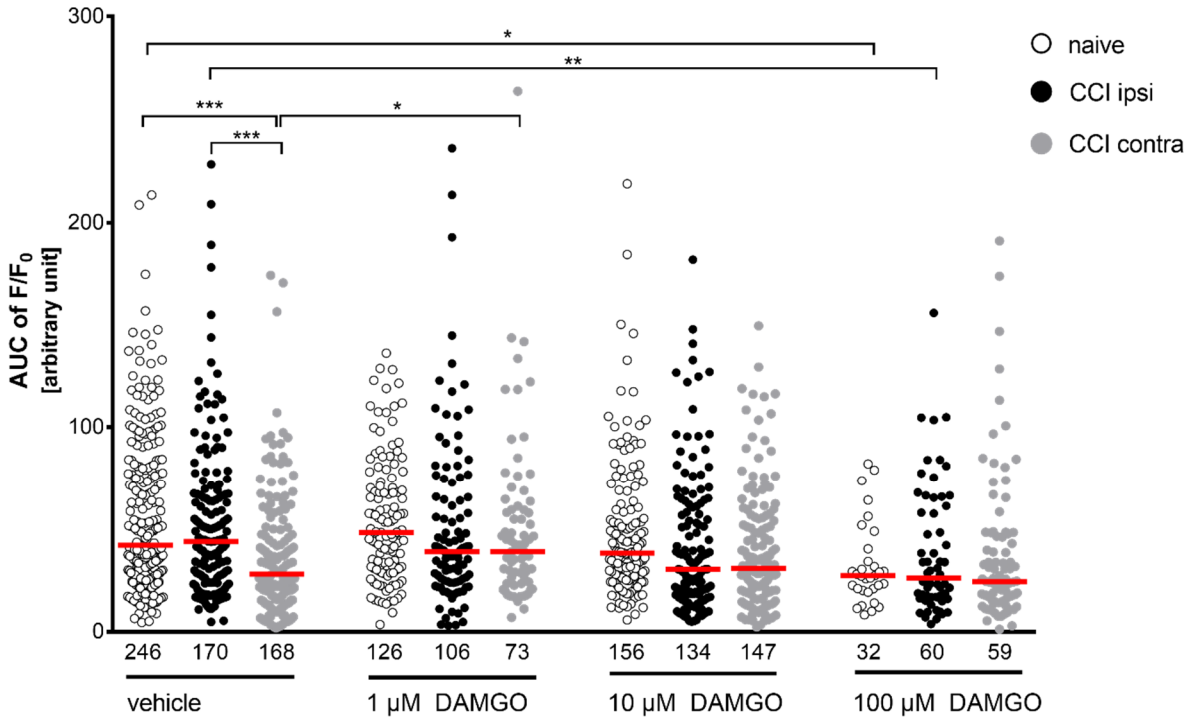

Figure 3.3: Effects of DAMGO on thallium uptake in mouse DRG neurons in the absence of K_v and K_{ATP} channel blockers, measured as increase in fluorescence intensity of the FluxOR dye. Data are quantified as area under the curve (AUC) of change in fluorescence intensity (F/F_0) after application of stimulus buffer containing either thallium and DAMGO (1-100 µM as indicated) or thallium alone (vehicle). Single dots represent single cells, bars indicate median. Numbers below indicate number of cells from at least 3 cultures per group, from 4 animals per culture (* $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$; Kruskal-Wallis one-way ANOVA followed by Dunn's test. See Supplementary Table 1 for details).

Taken together, thallium uptake, which reflects potassium conductance, was not increased by DAMGO treatment in DRG cells from naïve or CCI mice. A high DAMGO dose (100 µM) rather lowered the conductance. Thus, CCI did not result in enhanced DAMGO-induced potassium flux in mouse DRG neurons. These findings were unexpected and prompted questions regarding validity of the FluxOR method.

3.1.3. No Change in $K_{ir}3$ Channel Conductance by DAMGO in HEK 293 Cells Transfected with MOR and $K_{ir}3.2$

For validation of the FluxOR assay, HEK 293 cells transfected with plasmids encoding MOR and $K_{ir}3.2$ were used as a positive control. TEA and glibenclamide were used to inhibit effects mediated by K_v or K_{ATP} channels, respectively, to specifically investigate $K_{ir}3.2$-mediated effects. Since in the above experiments 1 µM DAMGO was ineffective and 100 µM inconclusive, in these experiments we tested the standard DAMGO dose of 10 µM.

Application of stimulus buffer containing DAMGO (10 µM or vehicle) and thallium after 20 s of base-line recording resulted in increased fluorescence in all cells (Figure 3.4A). Nevertheless, treatment with DAMGO (10 µM) did not enhance thallium uptake compared to vehicle treatment (Figure 3.4B), indicating no functional coupling of MOR and $K_{ir}3.2$ channels, when employing the FluxOR Potassium Imaging Assay. These results were unexpected and are discussed in section 4.1.4.

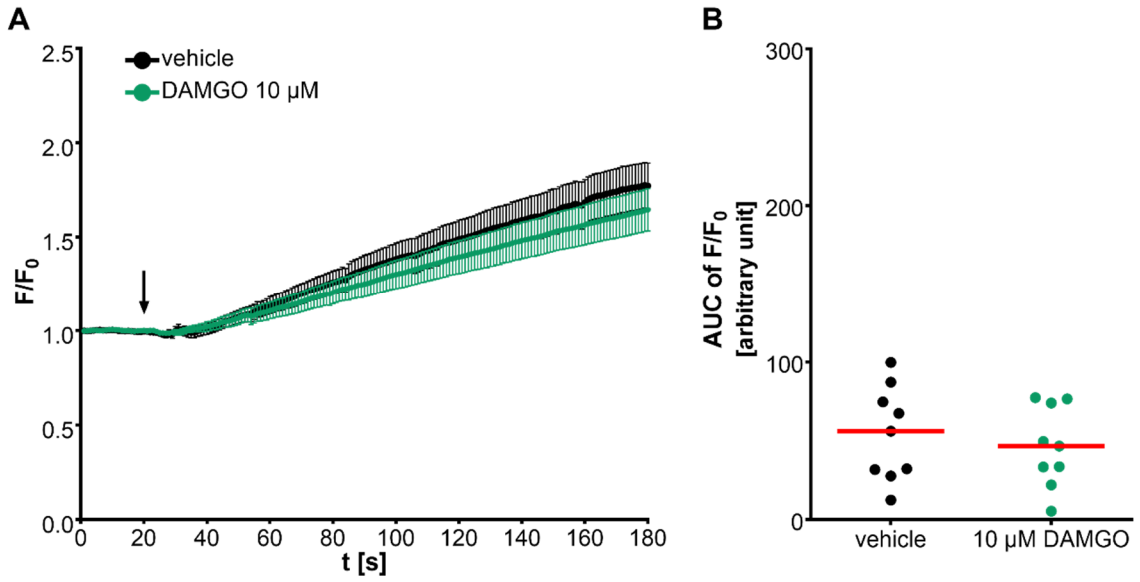

Figure 3.4: Effects of DAMGO on thallium uptake in HEK 293 cells transfected with MOR and $K_{ir}3.2$ in the presence of K_v and K_{ATP} channel blockers, measured as increase in fluorescence intensity of the FluxOR dye. (A) Traces showing change in fluorescence over time. Fluorescence, indicating thallium uptake, increased upon addition of stimulus buffer containing thallium and 10 µM DAMGO or thallium alone (vehicle). The buffer also contained blockers of K_v (TEA) and K_{ATP} (glibenclamide) channels. Arrow indicates application of the buffers. Mean and SEM are shown for each time point. Means were calculated from all technical replicates (i.e. n = 9 plates). (B) Data shown in A, quantified as area under the curve (AUC) of change in fluorescence intensity (F/F_0) after application of stimulus buffer. Single dots represent technical replicates, i.e. plates recorded, bars indicate means. ($p = 0.5582$; unpaired t-test. N = 9 plates in both groups, sampled from 3 transfections. Total number of recorded cells per group: 702 (vehicle) and 863 (DAMGO)).

3.2. Patch Clamp Recordings to Investigate $K_{ir}3$ Channel Modulation by MOR-Agonist DAMGO in DRG Neurons of Naïve and CCI Mice

Since FluxOR Potassium Imaging experiments were not sufficiently informative to answer our questions, we decided to employ a more established method to investigate the effect of CCI on DAMGO-induced potassium channel activity in murine DRG neurons.

3.2.1. DAMGO induces $K_{ir}3$ Currents in HEK 293 Cells Transfected with MOR and $K_{ir}3.2$

To establish the protocol for patch clamp recordings of inwardly rectifying potassium currents, HEK 293 cells transfected with MOR and $K_{ir}3.2$ cDNA and untransfected (control) HEK 293 cells were used. Effects of DAMGO (10 µM) on HEK 293 cells were recorded in whole cell voltage clamp mode at constant holding potential of -80 mV. Cells were considered responders to DAMGO application if the resulting current was larger than three times the noise range (see section 2.4.6). According to this criterion, most of the cells in the transfected group showed prompt and reversible (by washout or addition of $BaCl_2$) inward current upon DAMGO application (see Figure 3.5 for exemplary traces), while in the untransfected group only one cell showed very small, questionable response to DAMGO (Figure 3.5D, discussed in section 4.2.1).

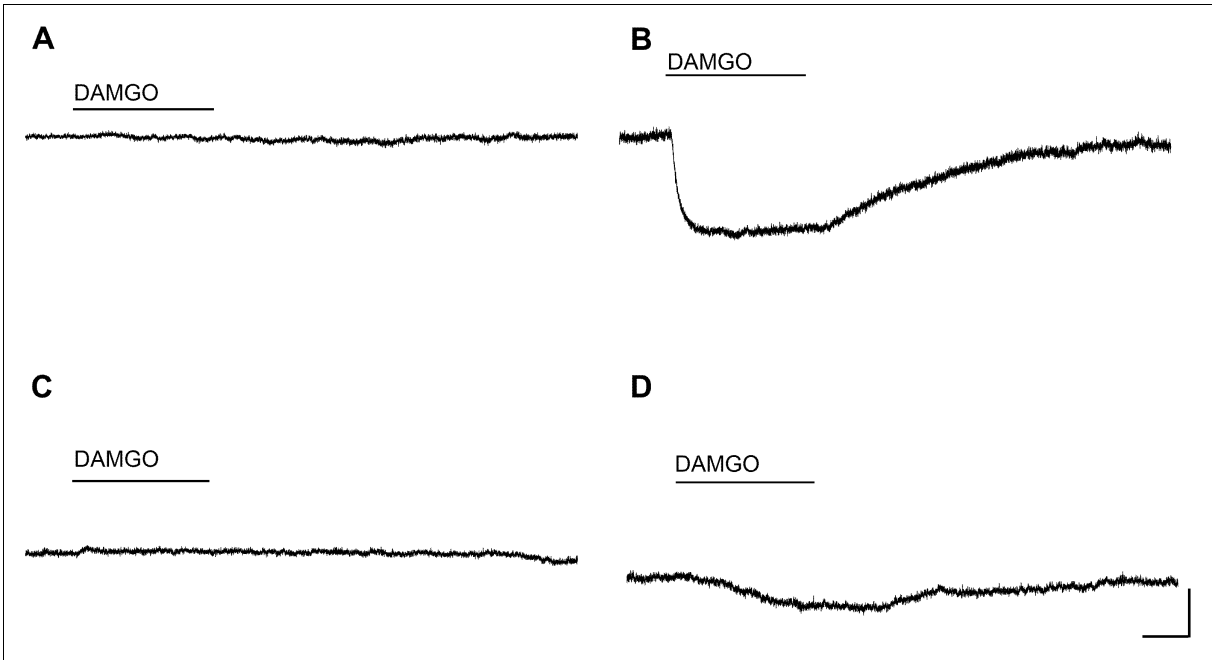

Figure 3.5: Exemplary traces of DAMGO-induced inward currents in hyperpolarized HEK 293 cells. (A, B) Traces of DAMGO-non-responder (A) and DAMGO-responder (B) among HEK 293 cells transfected with MOR and $K_{ir}3.2$. (C) Trace of DAMGO-non-responder among untransfected HEK 293 cells. (D) Trace of the one untransfected HEK 293 cell by definition classified as responding to DAMGO. DAMGO was used in a concentration of 10 µM. Currents were recorded in voltage clamp mode at -80 mV in high K^+ extracellular buffer (140 mM). Scale bar: 10 s, 200 pA.

Analysis of all recorded HEK 293 cells revealed a significantly higher rate of DAMGO-responders in transfected than in untransfected cells (Figure 3.6). These results clearly show functional coupling of MOR and $K_{ir}3.2$ in transfected HEK 293 cells.

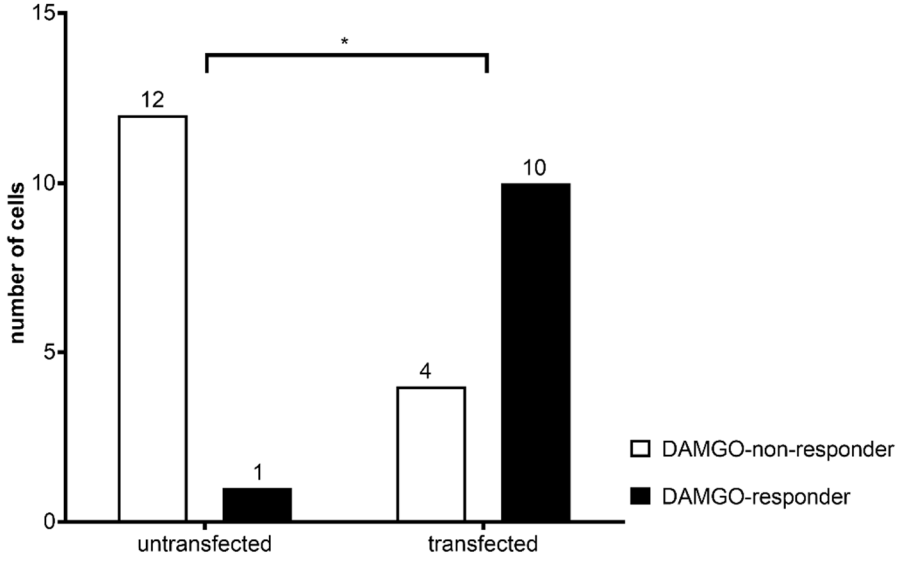

Figure 3.6: Functional coupling of MOR and $K_{ir}3.2$ channels in transfected HEK 293 cells. The graph shows the number of cells (stated above bars) responding and non-responding to DAMGO (10 µM) in untransfected HEK 293 cells and HEK 293 cells transfected with MOR and $K_{ir}3.2$. The proportion of DAMGO-responders to DAMGO-non-responders was significantly higher in transfected compared to untransfected cells (* $p = 0.0013$; Fisher's exact test).

Assessment of the currents in all cells recorded revealed that application of DAMGO to transfected cells resulted in significantly larger inward currents than in untransfected cells (Figure 3.7A). Inward currents of comparable size were measured in transfected HEK 293 cells when only DAMGO-responding cells were analyzed (Figure 3.7B), These findings indicate functional coupling of MOR and $K_{ir}3.2$ in transfected HEK 293 cells.

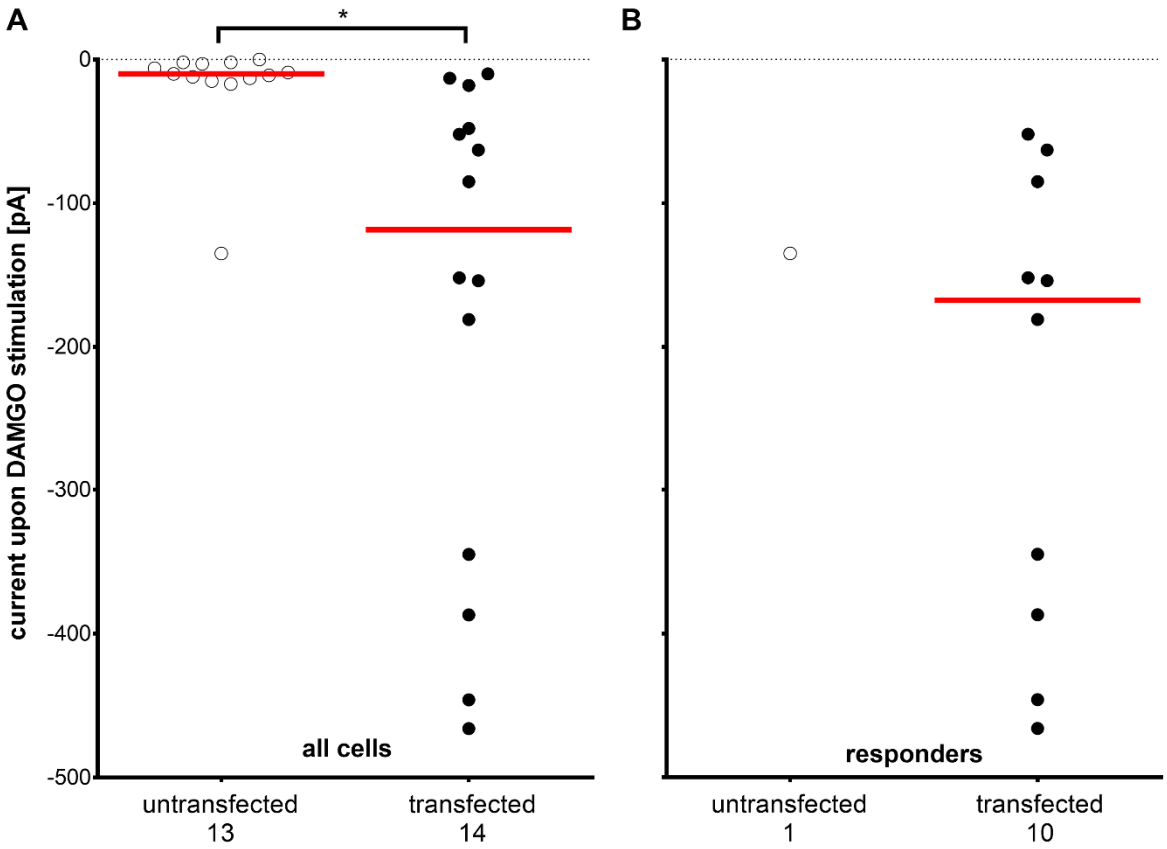

Figure 3.7: DAMGO-induced inward currents in hyperpolarized HEK 293 cells. Experiments were performed in untransfected HEK 293 cells and HEK 293 cells transfected with MOR and $K_{ir}3.2$. Currents were recorded in voltage clamp mode at holding potential -80 mV in high K^+ extracellular buffer (140 mM). (A) Data points represent DAMGO (10 µM) -induced single cell currents of all cells recorded, including DAMGO-responding and non-responding cells. Resulting inward currents were significantly larger in transfected than in untransfected HEK 293 cells (* $p < 0.0001$; Mann-Whitney U test). (B) The same data as in (A) only showing cells responding to DAMGO. Cells were defined as responding to DAMGO if the resulting current was larger than three times the noise range. Data points represent single cell values, bars indicate medians. Numbers below indicate number of cells, sampled from at least 3 cultures/transfections per group.

Application of the potassium channel blocker $BaCl_2$ reversed DAMGO-mediated currents in DAMGO-responders in transfected HEK 293 cells (Figure 3.8B), indicating that inward currents were mediated by potassium channels. The one untransfected HEK 293 cell which by definition was classified as responding to DAMGO (Figure 3.8A) also showed $BaCl_2$-mediated reversal of the current upon DAMGO application.

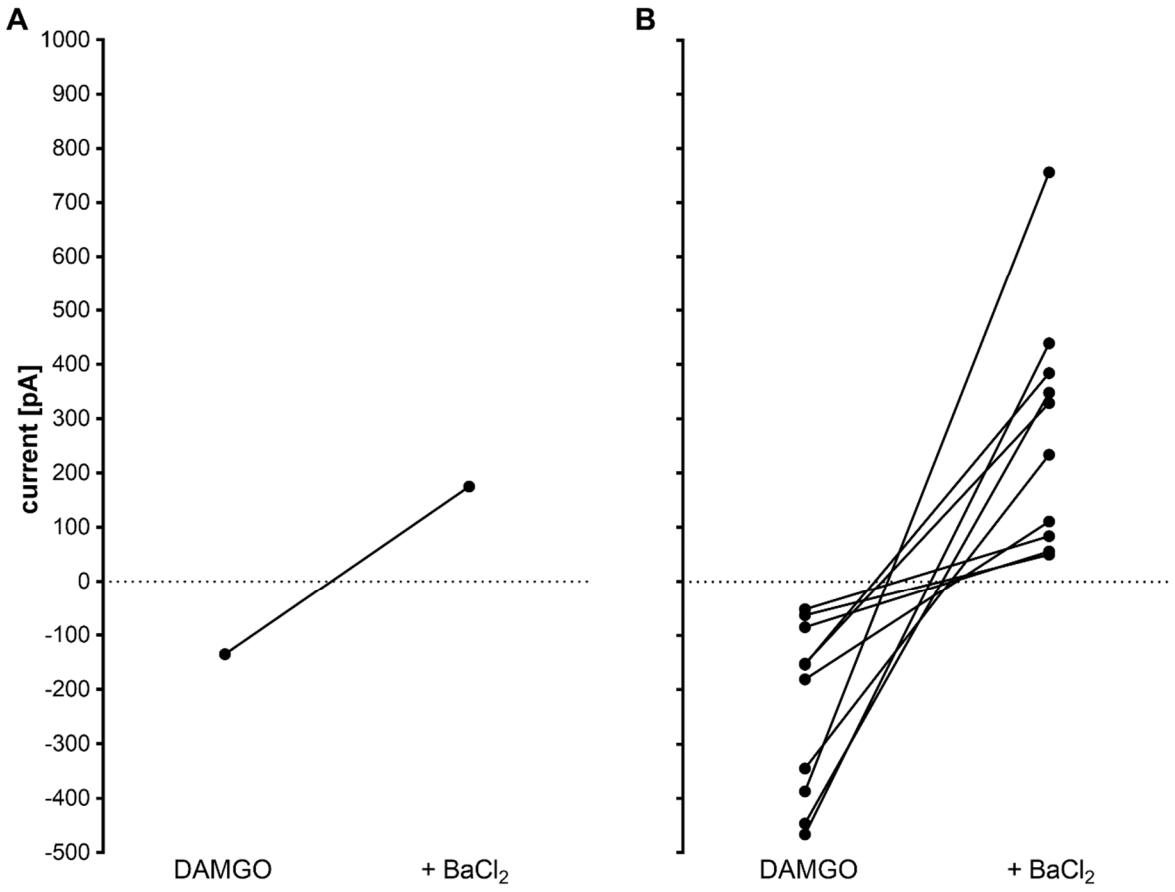

Figure 3.8: $BaCl_2$-mediated reversibility of inward currents induced by DAMGO in HEK 293 cells. Data points represent DAMGO (10 µM) induced whole cell currents of the same cell before and after application of $BaCl_2$ (3 mM). Currents were recorded in voltage clamp at holding potential -80 mV in high K^+ extracellular buffer (140 mM). Only DAMGO-responding cells are shown. (A) Effects in untransfected HEK 293 cell (n = 1). (B) Effects in HEK 293 cells transfected with MOR and $K_{ir}3.2$ (n = 10). Dotted lines represent zero current.

3.2.2. DAMGO induces Potassium Currents in DRG Neurons of Naïve and CCI Mice

After establishing that DAMGO-induced inward currents through $K_{ir}3.2$ channels could be successfully recorded in MOR and $K_{ir}3.2$ transfected HEK 293 cells, in the following experiments possible effects of nerve injury on potassium conductance in mouse DRG neurons were investigated using whole-cell patch clamp recordings. Effects of DAMGO (10 µM) on neurons from DRGs ipsilateral to the CCI and neurons from DRGs of naïve mice were recorded in whole cell voltage clamp mode at constant holding potential of -80 mV. In both naïve mice and mice 2 days following CCI, there were DRG cells showing prompt inward current upon DAMGO application, which could be reversed by $BaCl_2$ (see Figure 3.9 for exemplary traces).

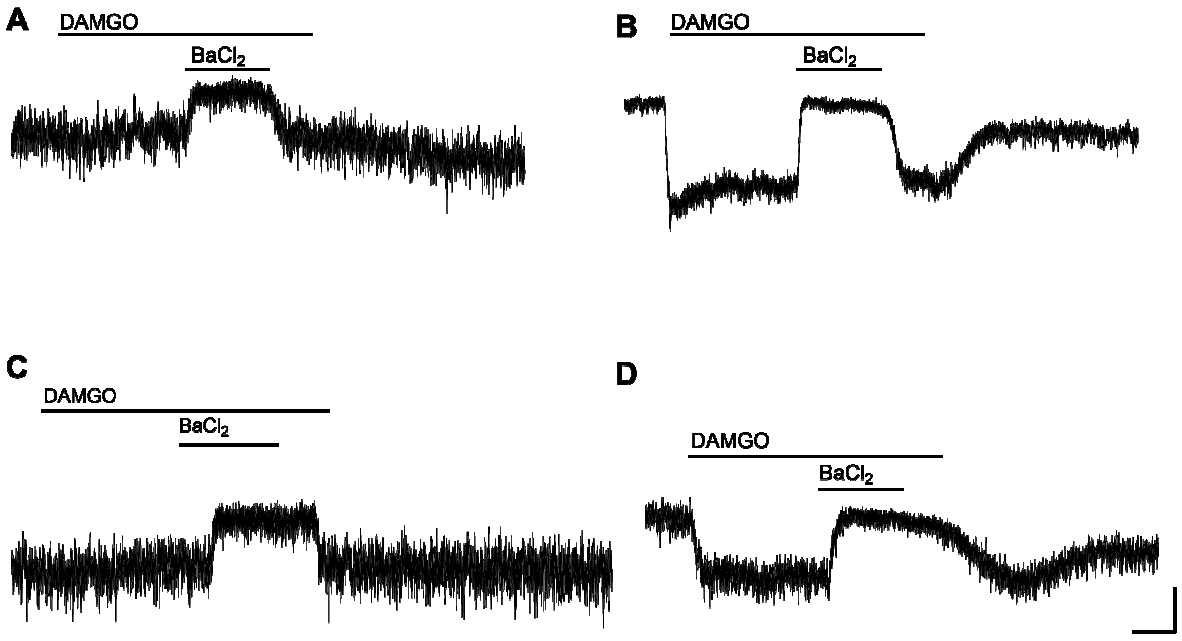

Figure 3.9: Exemplary traces of DAMGO-induced inward currents in hyperpolarized mouse DRG neurons. Effects of DAMGO (10 µM) and $BaCl_2$ (3 mM) on whole-cell current are shown. Recordings were obtained in voltage clamp mode at -80 mV in high K^+ extracellular buffer (140 mM). (A, B) Traces of mouse DRG neurons 2d following CCI non-responding (A) and responding (B) to DAMGO, before and after $BaCl_2$ application. (C, D) Traces of mouse DRG neurons in naïve mice non-responding (C), responding (D) to DAMGO, before and after $BaCl_2$ application. Scale bar: 10 s, 200 pA

Although there was a tendency for higher percentage of DAMGO-responding cells in DRGs from naïve mice (15%) than from mice exposed to CCI (4%), the rate of DAMGO-responding cells between naïve and CCI mice was not statistically significant (Figure 3.10).

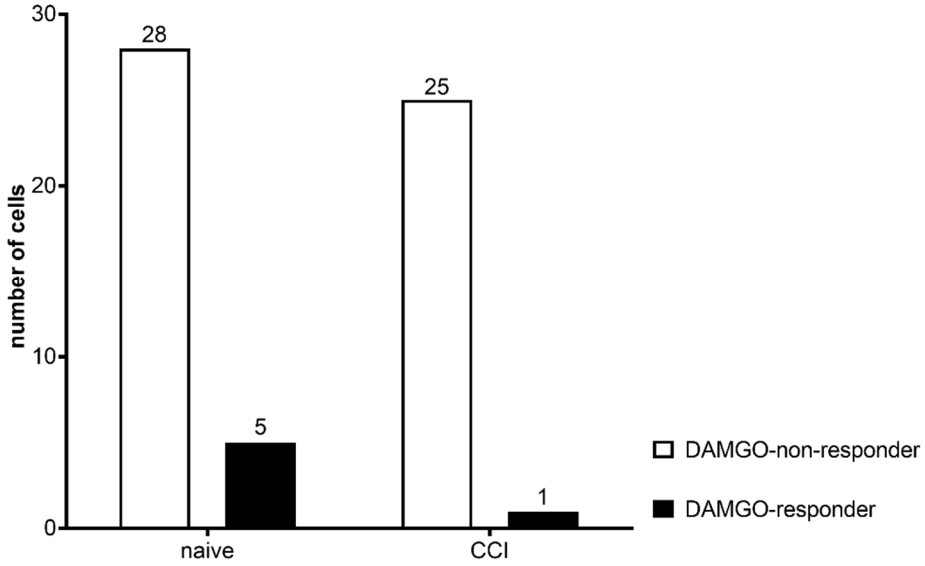

Figure 3.10: No effect of nerve injury on the rate of DAMGO-responding DRG mouse neurons. The graph shows the number of cells (stated above bars) responding and non-responding to DAMGO (10 µM) in DRG neurons from naïve mice and mice exposed to CCI. The proportion of DAMGO-responding to DAMGO-non-responding neurons in naïve versus CCI mice did not differ significantly ($p = 0.2148$; Fisher's exact t-test).

Assessment of the currents in all recorded cells revealed that DAMGO-induced inward currents were significantly smaller in DRG neurons from mice exposed to CCI compared to naïve mice (Figure 3.11A). Separate analysis of DAMGO-responding cells revealed small inward currents upon DAMGO treatment in both naïve as well as CCI group (Figure 3.11B).

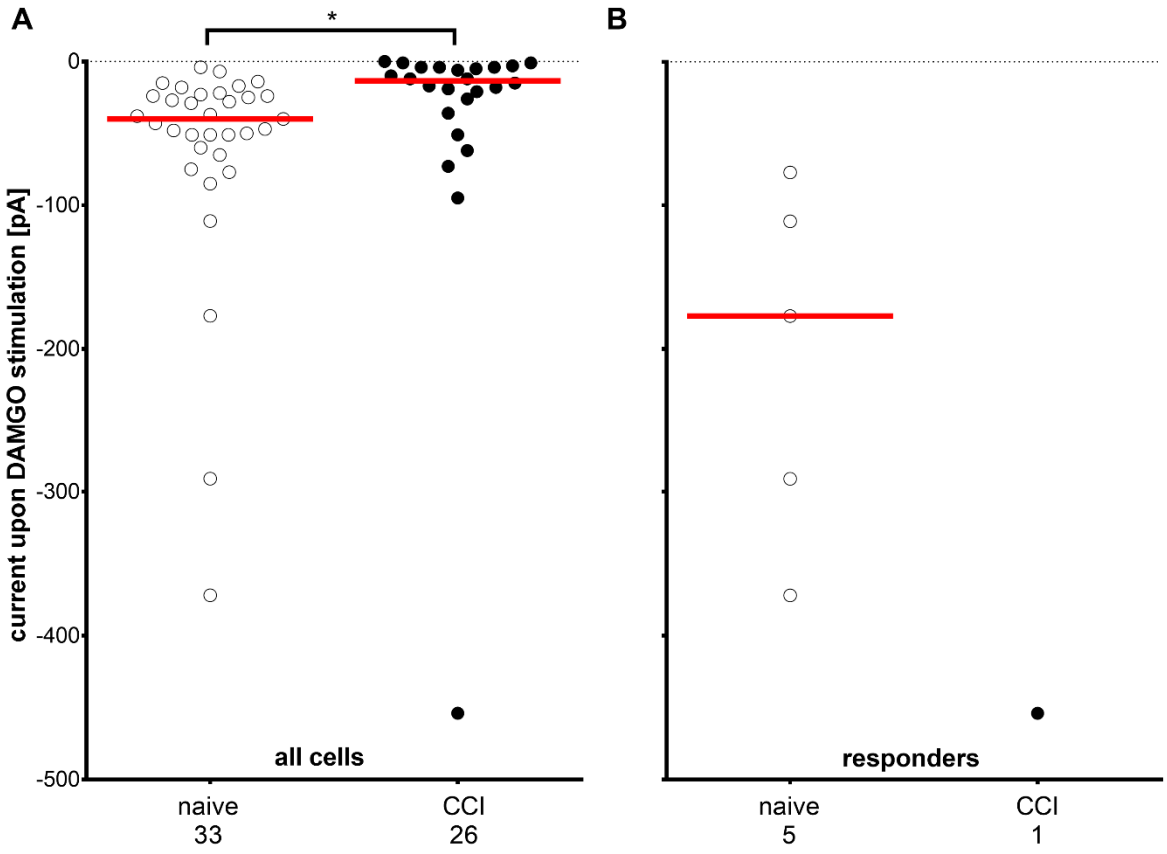

Figure 3.11: Effects of nerve injury on DAMGO-induced inward currents in hyperpolarized mouse DRG neurons. Experiments were performed in neurons from naïve mice and mice exposed to CCI. Currents were recorded in voltage clamp mode at -80 mV in high K^+ extracellular buffer (140 mM). (A) Data points represent DAMGO (10 µM) -induced single cell currents of all cells recorded, including DAMGO-responding and non-responding cells. Resulting inward currents were significantly smaller in neurons from CCI group than compared to naive. (* p = 0.0009; Mann-Whitney U test). (B) The same data as in (A) only showing cells responding to DAMGO. Cells were defined as responding to DAMGO if the resulting current was larger than three times the noise range. Data points represent single cell values, bars indicate median. Numbers below indicate number of cells, sampled from at least 3 cultures per group.

These data suggest the activation of potassium channels by DAMGO, which resulted in inward currents in hyperpolarized state in murine DRG neurons under naïve and CCI conditions. They also show that CCI does not result in increased effect of DAMGO on potassium currents in mouse DRG neurons.

Application of the potassium channel blocker $BaCl_2$ reversed DAMGO-mediated currents in DAMGO-responders from both naïve (**Figure 3.12**A) and CCI mice (**Figure 3.12**B), indicating that inward currents were mediated by potassium channels.

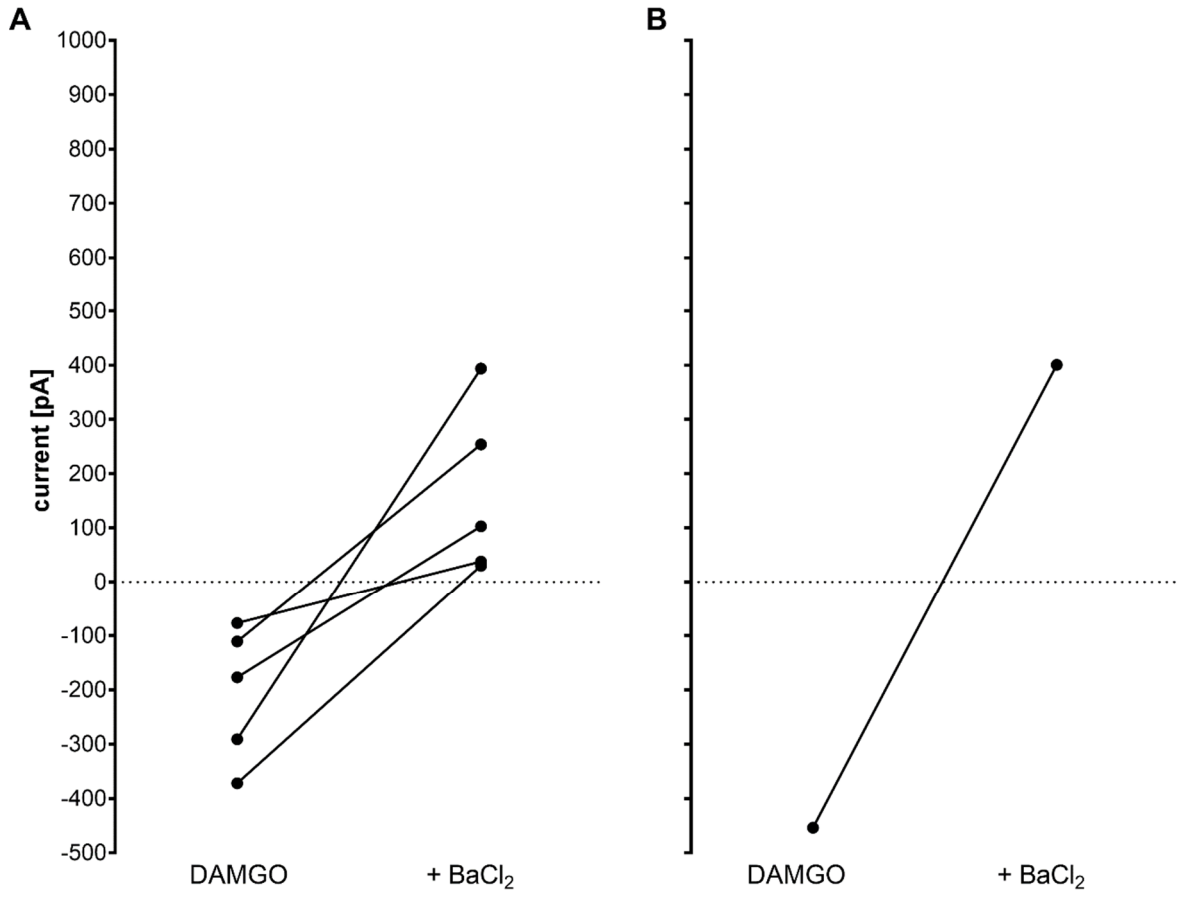

Figure 3.12: $BaCl_2$-mediated reversibility of inward currents induced by DAMGO in mouse DRG neurons. Data points represent DAMGO (10 µM) -induced whole cell currents of the same cell before and after application of $BaCl_2$ (3 mM). Currents were recorded in voltage clamp at holding potential -80 mV in high K^+ extracellular buffer (140 mM). Only DAMGO-responding cells are shown. (A) Effects in DRG neurons from naïve mice (n = 5). (B) Effects in DRG neurons from CCI mouse (n = 1). Dotted lines represent zero current.

4. Discussion

The main objective of this project was to examine the impact of peripheral nerve injury on potassium conductance following application of MOR agonist DAMGO in murine primary afferent neurons.

I found that DAMGO induced potassium currents in DRG neurons of both naïve mice and mice exposed to CCI of the sciatic nerve. However, the CCI did not result in increased K_{ir} channel conductance upon application of DAMGO. In fact, our electrophysiology data show a tendency towards smaller current following CCI.

4.1. Real Time Fluorescence Microscopy to Monitor Modulation of Potassium Channel Conductance by MOR Agonist DAMGO

Traditional approaches to investigate ion channel physiology usually employ single cell recordings, often using the patch clamp technique. However, these single cell approaches are time-consuming, as only one cell can be recorded at a time. Since the effects of nerve injury on opioid-mediated potassium conductance in primary afferent neurons have not been examined so far, we first opted for high-throughput approach employing the FluxOR potassium imaging assay to investigate in potassium channel conductance in response to DAMGO application in mouse DRG neurons. This technique allows for the recording of up to 30 – 100 cells simultaneously. Ion selectivity of the fluorogenic dye and the use of channel blockers in the assay buffer allow to selectively investigate specific ion channel function in this assay.

4.1.1. Targeting $K_{ir}3$ Channels - No Change in $K_{ir}3$ Channel Conductance upon DAMGO Application in DRG Neurons of Naïve and CCI Mice

Main targets of our investigations were $K_{ir}3$ channels as these have recently been implicated as major contributors to peripheral opioid analgesia (Nockemann et al., 2013). To specifically investigate conductance mediated by $K_{ir}3$ channels, TEA and glibenclamide were used to block and thus exclude effects mediated by K_v and K_{ATP} channels, known to be present in mouse DRG neurons (Cunha et al., 2010; Tsantoulas et al., 2012; Manteniotis et al., 2013). Under these conditions, DAMGO did not change potassium conductance in DRG neurons from either naïve or CCI mice, suggesting no involvement of $K_{ir}3$ channels (Figure 3.2). The effects in naïve mice are in agreement with published literature, as functional $K_{ir}3$ channels were not detected in

mouse DRG neurons (Kanjhan et al., 2005; Nockemann et al., 2013) and opioids do not produce analgesia via peripheral opioid receptors in the absence of tissue injury (Stein et al., 1989; Stein and Machelska, 2011; Labuz and Machelska, 2013).

However, the findings in CCI mice were somewhat unexpected, since $K_{ir}3$ channels have been implied as important mediators of peripheral opioid analgesia upon tissue damage (Nockemann et al., 2013), and activation of peripheral opioid receptors ameliorated CCI-induced mechanical and heat hypersensitivity in mice (Kolesnikov et al., 2007; Hervera et al., 2012; Labuz and Machelska, 2013; Labuz et al., 2016). Hence, the enhanced functional coupling of $K_{ir}3$ channels and MOR in primary afferent neurons after CCI was assumed as a possible mechanism underlying the observed analgesic effects mediated by peripheral MOR in CCI mice.

4.1.2. Targeting Potassium Channels - No Enhancement of Potassium Channel Conductance upon DAMGO Application in DRG Neurons of Naïve and CCI Mice

Since the lack of DAMGO effect on $K_{ir}3$ channel conductance was somewhat surprising, we next examined whether other potassium channels may be involved. Therefore, in the subsequent experiments we omitted blockers of K_v and K_{ATP} channels.

Nevertheless, the omission of K_v and K_{ATP} channel blockers also did not result in increased potassium channel conductance upon DAMGO application in DRG neurons of both naïve and CCI mice. Actually, higher DAMGO dose (100 µM) decreased potassium channel conductance in DRG cells of naïve mice and in cells of DRGs ipsilateral to CCI when compared to vehicle treatment (Figure 3.3). It is rather unlikely that inherent optical properties of DAMGO itself, i.e. absorption of light by higher concentrations, underlie this observation, since comparable effects were not seen in the experiments using DAMGO (100 µM) and K_v and K_{ATP} channel blockers (Figure 3.2). Other factors excluded, a possible explanation might be the reduced potassium flux through calcium sensitive potassium channels due to inhibition of calcium channels by opioids, as reported for morphine in rat DRG neurons (Abdulla and Smith, 1998).

An additional surprising result was the lower potassium channel conductance in vehicle-treated neurons from DRGs contralateral to CCI. This result could suggest a lower expression or function of potassium channels or MOR in DRG neurons contralateral to CCI, leading to reduced basal potassium conductance upon vehicle treatment. While changes in expression and activity of ion channels and receptors contralateral to the site of a nerve lesion cannot be excluded, MOR expression and mechanical sensitivity in primary afferent neurons contralateral

to a lesion have thus far been shown to been unaffected by CCI (Labuz et al., 2009; Obara et al., 2009; Lee et al., 2011; Labuz and Machelska, 2013). Additionally, since this effect was not seen in any other treatment group in my experiments, it his highly unlikely to be a result of the CCI itself, but could possibly reflect the inconsistency of the assay, as discussed in section 4.1.4.

4.1.3. Validation of the Potassium Imaging Method in Transfected HEK 293 cells

Since $K_{ir}3$ channels have been shown to functionally couple to opioid receptors allowing opioids to enhance potassium conductance (Kobayashi et al., 2006; Nockemann et al., 2013; Nagi and Pineyro, 2014), we decided to use HEK 293 cells transfected with $K_{ir}3.2$ and MOR as a model to validate the potassium imaging method. Surprisingly, DAMGO treatment did not result in increased potassium channel activity compared to vehicle treatment in these cells (Figure 3.4), indicating no effect of DAMGO on potassium flux through $K_{ir}3.2$ channels. Since this is in conflict with our assumption and published literature (see above), these results raised concerns regarding suitability of the FluxOR imaging approach for our experiments.

4.1.4. Concerns with the FluxOR Imaging Approach

Cell lines like HEK 293 are well suited for approaches relying on homogenous cell suspensions recorded in plate readers with high numbers of technical replicates of highly homogeneous samples. Manual selection of single cells in our experiments could account for high response variability between technical replicates (Figure 3.4), possibly aggravated by varying rate of successful co-transfection. These factors, thus, cast doubt on the ability of the assay to produce consistently reproducible data in our experimental approach. This is even more challenging in case of DRG neuron cultures, which in contrast to cell lines, are very heterogeneous regarding cell subpopulations (C, Aδ and Aβ neurons) and their respective expression of ion channels and receptors. Therefore, investigation of dissociated neuron cultures requires selection of single cells based on cellular characteristics such as morphology and size. Importantly, proper analysis of effects in specific neuronal subpopulations depends on correct selection of cells, which can prove demanding. Lack of suitable tools for selective labeling of different cell populations in our experimental approach could account for insufficient distinction of cell types and high response variability (Figure 3.3). Identification of effects mediated by the system of interest may be difficult, as they can be hidden among larger, unspecific effects. For example, large potassium background conductance could be a possible reason for difficulties in our

experiments. Thus, although FluxOR has previously been employed to study native neuronal cultures (Nockemann et al., 2013), the methodological difficulties discussed above might account for variability among the studies.

An additional caveat is, that this method does not permit to control the membrane potential of recorded cells. Additionally, since the manufacturer does not disclose the full composition of the buffers, it is not possible to determine or at least estimate the membrane potential of the cells by use of Goldman-Hodgkin-Katz equation or any similar approach. Thus, the assay does not permit to determine the state of ion channels with voltage dependent gating properties such as K_v or $K_{ir}3$ channels. The patch clamp technique in contrast permits tight control of electrophysiological cellular parameters like membrane potential as well as precise cell selection guided by morphology and electrophysiological properties.

4.2. Patch Clamp Recordings to Investigate $K_{ir}3$ Channel Modulation by MOR Agonist DAMGO

4.2.1. DAMGO-induced $K_{ir}3$ Currents in HEK 293 Cells Transfected with MOR and $K_{ir}3.2$

Using the whole-cell voltage clamp approach, DAMGO induced inward currents could successfully be recorded from hyperpolarized HEK 293 cells transfected with MOR and $K_{ir}3.2$ cDNA (Figure 3.5). The rate of cells responding to DAMGO was significantly higher and the DAMGO-induced inward currents were significantly larger in transfected cells compared to untransfected cells (Figure 3.7). Furthermore, the reversibility of DAMGO-induced currents by potassium channel blocker $BaCl_2$ indicates that currents were indeed mediated by potassium channels (Figure 3.8).

Among the untransfected cells, one cell had to be classified as DAMGO responder (Figure 3.5D) according to the criterion that the DAMGO-mediated current is three times larger than the noise range (see section 2.4.6). However, the kinetics of this particular cell response (slow, no distinct onset correlated to stimulus, very small amplitude) neither resembles the kinetics of transfected cells' responses (fast, distinct onset tightly correlated to stimulus application, prompt return to baseline) nor the responses seen in the literature (Kohno et al., 2005; Kobayashi et al., 2006;

Nockemann et al., 2013; Gorham et al., 2014). Thus, judged by the response kinetic, this cell does not appear to reliably respond to DAMGO.

Substantially higher ratio of DAMGO-responders in MOR and $K_{ir}3.2$ transfected compared to untransfected HEK 293 cells and $BaCl_2$ reversibility clearly demonstrate successful identification of DAMGO-induced inward currents and suggest they were mediated by $K_{ir}3.2$ channels.

4.2.2. DAMGO-induced Potassium Currents in DRG Neurons of Naïve and CCI Mice

After successfully establishing the patch clamp protocol in transfected HEK 293 cells, DRG neurons from naïve and CCI mice were investigated. DAMGO-induced potassium currents could be recorded in DRG neurons from naïve and CCI mice (Figure 3.9 and Figure 3.11). DAMGO-induced currents were reversed by application of the potassium channel blocker $BaCl_2$ (Figure 3.12).

The numbers of DAMGO-responding cells were insufficient to statistically compare the size of their currents between naïve and CCI mice (Figure 3.11) Although there was a tendency for lower percentage of DAMGO-responding cells in DRGs from CCI mice (4%, 1 out of 26 cells) than from naïve mice (15%, 5 out of 33 cells), the ratio of DAMGO-responders between naïve and CCI mice was not statistically different (Figure 3.10).

In contrast to our hypothesis, CCI to the sciatic nerve did not enhance DAMGO-induced potassium channel conductance in mouse DRG neurons. On the contrary, mean inward current upon DAMGO application was smaller in the CCI group than in the naïve group (Figure 3.11).

$K_{ir}3$ Channels in Naïve Mouse DRG Neurons

However, it is somewhat intriguing, that we did record any DAMGO-induced currents from hyperpolarized mouse DRG neurons (Figure 3.9, 3.10, 3.11, 3.12). Whereas $K_{ir}3$ channels were identified as important mediators of peripheral opioid analgesia in rats (Gao et al., 2007; Nockemann et al., 2013; Chung et al., 2014), few publications so far investigated $K_{ir}3$ channel expression and functioning in primary afferent neurons of the mouse, and the data appear inconclusive:

Nockemann et al. (2013) showed very low amounts of $K_{ir}3.1$ and $K_{ir}3.2$ mRNA transcripts and no immunoreactivity for the corresponding protein products in mouse DRGs. Using patch

clamp recordings, the authors reported “negligible” inward currents upon DAMGO application and concluded on the absence of $K_{ir}3$ from mouse DRG neurons. However, this interpretation of their patch clamp data could be challenged since the size of DAMGO-induced currents in naïve mouse DRG neurons appears substantial (1.8±0.4 nA) (Nockemann et al., 2013) when compared to currents recorded under similar conditions reported as opioid-mediated responses in rat DRG and spinal cord neurons, or Xenopus oocytes transfected with $K_{ir}3$ channels (40-800 pA) (Kohno et al., 2005; Kobayashi et al., 2006; Gao et al., 2007).

Kanjhan et al. (2005) reported lack of hyperpolarization-activated potassium currents characteristic for $K_{ir}3$ using patch clamp in DRG neurons of P0 mice. The authors argued that $K_{ir}3$ expression might occur later in the development of the nervous system, but did not show the corresponding data from older animals.

Mitrovic et al. (2003) stated a lack of $K_{ir}3.2$ immuno-staining in mouse DRGs, but did not present the corresponding data.

In a comprehensive RNA expression analysis of mouse sensory ganglia, Manteniotis et al. (2013) reported intermediate expression levels of KCNJ3 mRNA coding for $K_{ir}3.1$ in DRG and trigeminal ganglia. $K_{ir}3.1$ and $K_{ir}3.2$ are known to be frequently co-expressed and form functional heterotetramers in neurons. In the $K_{ir}3$ channel family, only $K_{ir}3.2$ is known to be able to form functional homotetramers (Lüscher and Slesinger, 2010). Thus, the expression of $K_{ir}3.1$ alone is not sufficient to form a functional channel.

Interestingly, however, in a recently published study, mRNAs encoding $K_{ir}3.1$ and 3.2 were found in mouse DRGs, which would allow formation of functional heterotetrameric channels (Saloman et al., 2016).

Taken together, the current literature suggests low to intermediate $K_{ir}3$ mRNA expression (Nockemann et al., 2013; Saloman et al., 2016), which may result in low protein level difficult to be detected by immunostaining methods (Mitrovic et al., 2003; Nockemann et al., 2013) and functional analysis (Nockemann et al., 2013) in DRG neuron somata in mice. In contrast, I found small, but reliable DAMGO-induced inward currents in hyperpolarized somata of mouse DRG neurons, compatible with currents mediated by $K_{ir}3$ channels as seen in our HEK 293 cell experiments and in rat neurons and Xenopus oocytes in other studies (Kohno et al., 2005; Kobayashi et al., 2006; Gao et al., 2007).

Potassium Channels Mediating DAMGO-Induced Currents and Neuropathy

Although the patch clamp conditions used in my experiments (high $[K^+]$ in extracellular buffer, hyperpolarizing holding potential) are widely used to study $K_{ir}3$ channels (Kobayashi et al., 2006; Gao et al., 2007; Nockemann et al., 2013; Gorham et al., 2014), other potassium channels possibly mediating the observed effects need to be considered, since no ion channel blockers specific for $K_{ir}3$ channels were used in my experiments. This is because $K_{ir}3$ specificity of substances such as tertiapin-Q, which has been heralded as a specific blocker of $K_{ir}3$ channels (Jin and Lu, 1998; Kanjhan et al., 2005), has recently been questioned by other authors (Nockemann et al., 2013).

The DAMGO-mediated effects in my experiments are highly unlikely to be mediated by K_v channels, since hyperpolarization leaves them in a closed state. Additionally, they are not typically considered to be regulated by opioids (Ocaña et al., 2004).

K_{Ca} channels can be indirectly regulated by opioids via calcium channels. However opioids typically inhibit calcium channels, which leads to lower calcium levels and thus lesser likelihood for K_{Ca} channels to be opened (Abdulla and Smith, 2001). Hence, their contribution to the DAMGO-induced effects in our experiments is also unlikely.

On the other hand, K_{ATP} (also known as $K_{ir}6$) channels are known to be activated by opioids, to show inward rectification (Cunha et al., 2010). They belong to the group of inwardly rectifying potassium channels and have been implicated to be involved in antinociceptive effects of various opioids in chronic pain conditions, including neuropathic pain in rats (Ocaña et al., 1995; Rodrigues et al., 2005; Kawano et al., 2009; Zoga et al., 2010; Du et al., 2011). There is no indication, that K_{ATP} channels are expressed in mouse DRG neurons. Thus, their potential involvement in DAMGO-mediated currents is unclear.

Another group of candidates possibly contributing to DAMGO-induced inward currents are K_{2P} channels, which include TREK and TESK channels. K_{2P} channels have been reported to be activated by opioids (Cho et al., 2016; Devilliers et al., 2013). Their role following nerve injury is complex and different subtypes seem to be differently regulated. For example, many subtypes are expressed in mouse DRG on mRNA level (Manteniotis et al., 2013; Mathie and Veale, 2015) and some have been shown to be downregulated in rat DRGs (TRESK) after peripheral nerve axotomy (Tulleuda et al., 2011). In a recent study in mice, differential regulation of different TREK subtypes has been shown in a CCI model, with TREK-1 being upregulated while TREK-2 being downregulated (Han et al., 2016). The overall functional effect of these alterations is

yet to be determined. Another more recent study reports expression in healthy DRG neurons and reduction of protein and mRNA coding for $K_{2P}1.1$ in mouse DRG neurons after spinal nerve ligation (Mao et al., 2017). Hence, with their linear current-voltage relationship K_{2P} channels could be involved in DAMGO-mediated potassium currents in DRG cells in naïve mice in our experiments, but their contribution to DAMGO effects following CCI remains to be elucidated.

In summary, considering the literature discussed above, the design of my experiments (buffer composition, voltage clamp) and the kinetics of the recorded currents, my experiments show DAMGO-induced currents in somata of DRG neurons from naïve as well as CCI mice likely to be mediated by K_{ir} and / or K_{2P} channels.

Downregulation of Potassium Channels in Neuropathic Pain

In my experiments, currents recorded upon DAMGO application were not enhanced following CCI, but were rather found reduced (Figure 3.11A). This effect could not be confirmed in the sub-analysis of DAMGO-responding cells due to low cell numbers (Figure 3.11B). However, smaller currents following CCI may point at reduction in expression or activity of potassium channels in somata of DRG neurons. Many studies in mice and rats have shown peripheral reduction in expression or activity of potassium channels in models of neuropathic pain (reviewed in Du and Gamper, 2013). In a rat model of neuropathic pain, a study found evidence for downregulation of the K_{2P} family member TRESK after axotomy (Tulleuda et al., 2011) and a recently published study reported reduction of protein and mRNA coding for $K_{2P}1.1$ in mouse DRG neurons after spinal nerve ligataion (Mao et al., 2017). Also, mRNA coding for $K_{ir}3.1$ and $K_{ir}3.2$ and their immunostaining were reduced in DRG and spinal cord after spinal nerve axotomy in rats (Lyu et al., 2015). Mechanisms proposed to regulate this downregulation include epigenetic silencing of potassium channel genes by methylation (Laumet et al., 2015) and channel redistribution along the axon (Calvo et al., 2016).

Opioid Receptors on Mouse DRG Neurons and Neuropathy

While MOR are expressed in primary afferent neurons of mice (Manteniotis et al., 2013), MOR mRNA and protein were shown to be reduced in DRG cells in models of neuropathic pain in mice (Rashid et al., 2004; Zhou et al., 2014; Zhang et al., 2016) and rats (Kohno et al., 2005; Obara et al., 2009; Lee et al., 2011). These findings prompted discussion, since opioids were shown to be effective in inhibition of action potential initiation and propagation in peripheral afferent neurons of mice after CCI (Schmidt et al., 2012) as well as in counteracting hyperalgesia in a mouse model of neuropathic pain when injected at the CCI site (Cayla et al.,

2012; Labuz and Machelska, 2013; Labuz et al., 2016). Interestingly however, immunohistochemistry, revealed accumulation of MOR at the CCI site in rats (Truong et al., 2003) and mice (Schmidt et al., 2013), which can account for effective local opioid analgesia. This indicates enhanced axonal transport of proteins from the soma to the terminals and accumulation of opioid receptors at the site of injury.

Regardless of the expression levels, however, activity and coupling of opioid receptors in DRG neuron somata may be independently altered after nerve injury, and have thus been subject of our investigation. While Abdulla et al. have shown reduced effects of opioids on calcium and potassium conductance in DRG neuron somata after axotomy (1998), in the present study we for the first time present data on activity and coupling of opioid receptors to potassium channels in DRG neuron somata after CCI.

4.3. Outlook

4.3.1. DRG Neuron Subpopulations

As in the imaging experiments, selection of the appropriate cell is pivotal to achieve informative patch clamp recordings from DRG cells. While potassium imaging doesn't allow to determine active membrane properties such as action potential generation, electrophysiology enables us to identify and classify neurons not only by morphological criteria, but also by further criteria such as action potential shape and resting membrane potential (Waddell and Lawson, 1990; Djouhri et al., 1998; Fang et al., 2005). Electrophysiology experiments separately analyzing neuronal subpopulations (C, Aβ, Aδ) following nerve injury provided deeper insight into mechanisms underlying neuropathic pain (Schmidt et al., 2012). Traditional patch clamp experiments so far mainly rely on manual single cell analysis. Thus, reported cell numbers in the literature frequently are low and many authors do not even separately report numbers of responding versus non-responding cells following pharmacological treatment. In our experiments, careful selection of cells by application of predefined criteria (see section 2.4.6) and reporting all data including non-responding cells still enabled us to detect DAMGO-mediated potassium currents in naïve mice and a tendency toward lower number of DAMGO-responding DRG neurons following nerve injury. Larger scale studies, however, are needed to differentiate the observed effects by specific neuronal subpopulations.

4.3.2. Sites of Investigation: Soma versus Axon

While recording from dissociated DRG neurons is still common practice in electrophysiology, the contribution of axon versus soma to transmission of nociceptive signals remains controversial. Interesting data by Du et al. (2014) have shed light on the role of the soma of uninjured primary afferents in nociceptive transmission. Expression and functioning of a vast set of receptors and ion channels in DRG neurons have been shown to be altered by nerve injury (Zoga et al., 2010; Abdulla and Smith, 2001; Lyu et al., 2015). Axotomy due to preparation of dissociated neuron cultures (section 2.4.2) might thus bias experimental outcomes in electrophysiological experiments (Tulleuda et al., 2011).

Additionally, in view of possible accumulation of potassium channels and opioid receptors at the injured nerve trunk (section 4.2.2), electrophysiological recordings from the injury site following local DAMGO application, for example using in vitro skin-nerve preparations,

appear appealing. Other appealing strategies are in-vivo patch clamp recordings and in vivo recordings from the nerve injury site. These are particularly interesting since they would allow to record responses from cell bodies or damaged axons upon application of stimuli to the corresponding receptive fields (Fang et al., 2005). Nevertheless, these approaches are technically very challenging and also have limitations such as necessity of keeping animals under anesthesia, which would need to be considered for data interpretation.

4.3.3. Species Differences and Translational Aspects

In the 1990s, advent of transgenic technologies has led to a shift in animal models used in pain research favoring mice instead of the traditionally used rats (Barrett and Haas, 2016). Discoveries made in one species were readily applied to the other one without much regard for possible species differences. Mounting frustration with poor translation of animal research into clinical applications eventually prompted questions regarding choice of species in pain research (Mogil, 2009). These issues have fortunately gained more attention lately (e.g. Chu et al., 2013). Careful choice of animal species for experiments and attention to possible species differences (Rigaud et al., 2008) when applying results to other fields and species will be pivotal for successful translation of animal data into clinical applications.

Little is known so far on how findings regarding potassium channels in neuropathic pain in rodents relate to clinical aspects of neuropathic pain. Human profiles of potassium channel expression under healthy and neuropathic conditions would therefore be an important step in our understanding of these conditions. Genetic association studies in humans have revealed influence of polymorphisms in potassium channel genes on pain phenotypes such as the risk for developing chronic pain conditions or acute pain sensitivity (Bruehl et al., 2013), postoperative opioid requirement (Nishizawa et al., 2009), and opioid addiction (Lotsch et al., 2010). However, there is limited knowledge on the peripheral neuronal expression and function of potassium channels and their involvement in pathophysiology of neuropathic pain in patients. Thus, identification of key mediators of pain and analgesia in the peripheral nervous system of humans is crucial for successful translation of discoveries made in animal research into clinical applications. Clearly, since gaining mechanistic insights in pain generation and inhibition is very limited in human trials, studies in animal models are – for now – inevitable (Mogil et al., 2010).

4.3.4. Future Therapeutic Approaches

Worthwhile therapeutic strategies in the future might involve targeting potassium channels on primary afferents to diminish their excitability in neuropathic pain states. Use of direct selective openers of specific potassium channel types presents one such opportunity. There are known direct openers of K_{ATP} channels (diazoxide and pinacidil) (Du et al., 2011), several K_{2P} channels (riluzole) (Duprat et al., 2000), and K_v channels (retigabine) (Rogawski and Bazil, 2008). All of these channels have been implicated in the development or maintenance of neuropathic pain (Ocaña et al., 2004; Du and Gamper, 2013; Tsantoulas and McMahon, 2014; Busserolles et al., 2016). In contrast, no specific direct openers have been identified for $K_{ir}3$ channels, which were recently heralded as important mediators of peripheral pain control (see section 1.4). Development of direct openers for $K_{ir}3$ subunits expressed in the nervous system might provide novel treatment strategies for neuropathic pain. However, since $K_{ir}3$ subunits are widely distributed throughout many organs, including brain and heart (see section 1.3.2), indirect targeting of $K_{ir}3$ channels via GPCRs like opioid receptors by peripherally restricted opioids might provide a more fruitful approach with less side effects (Ikeda et al., 2002; Nockemann et al., 2013; Kanbara et al., 2014). Since channels and receptors can be relocated upon tissue damage (see section 4.3.2), careful examination of their redistribution along nociceptive pathways is important to identify the most relevant site of analgesic actions. For example, following peripheral nerve damage, opioid receptors and potassium channels accumulating at the injury site (Schmidt et al., 2013; Calvo et al., 2016) may represent a valuable target for novel peripherally acting opioid therapeutics (Spahn et al., 2017).

5. Conclusion

This study shows for the first time electrophysiological data indicating opening of potassium channels such as $K_{ir}3$ or K_{2P} by MOR agonist DAMGO in primary afferent neurons of naïve mice, without tissue damage, as well as in mice following CCI of the sciatic nerve.

While – contrary to our assumption – DAMGO-mediated potassium conductance in DRG neurons was not enhanced following nerve injury, the presence of opioid-mediated potassium currents (in our experiments) and $K_{ir}3$ mRNA (reported in literature) in DRG neuron somata suggests a possible contribution of $K_{ir}3$ to peripheral opioid-mediated analgesia in neuropathy in mice.

Currents recorded from DRG neuron somata in our experiments were small and we observed a tendency towards even smaller currents after CCI, which is consistent with reports of lower presence of potassium channels as well as opioid receptors in soma of DRG neurons after nerve injury. Channel and receptor relocation have been reported upon tissue damage and prompt for further examination of their expression and function along nociceptive pathways to identify the most relevant sites of analgesic actions. Further, site specific analysis of potassium conductance and opioid effects comparing terminals, axon and soma of primary afferent neurons are needed to clarify the role of K_{ir} channels and MOR in peripheral nerve injury and opioid analgesia.

This thesis provides new insight into the interaction of potassium channels and opioid receptors on murine peripheral sensory neurons in health and neuropathy, and important methodological considerations for further investigations into the site-specific contributions of potassium channels to peripheral opioid analgesia in neuropathic pain.

6. Supplementary Information

Kruskal-Wallis test		
	p value	**summary**
ANOVA	< 0.0001	***
Dunn's multiple comparisons post-hoc test		
	adjusted p value	**summary**
naive vehicle *vs.* naive 1 µM DAMGO	> 0.9999	ns
naive vehicle *vs.* naive 10 µM DAMGO	> 0.9999	ns
naive vehicle *vs.* naive 100 µM DAMGO	0.0087	**
CCI ipsi vehicle *vs.* CCI ipsi 1 µM DAMGO	> 0.9999	ns
CCI ipsi vehicle *vs.* CCI ipsi 10 µM DAMGO	0.0875	ns
CCI ipsi vehicle *vs.* CCI ipsi 100 µM DAMGO	0.0348	*
CCI contra vehicle *vs.* CCI contra 1 µM DAMGO	0.0405	*
CCI contra vehicle *vs.* CCI contra 10 µM DAMGO	> 0.9999	ns
CCI contra vehicle *vs.* CCI contra 100 µM DAMGO	> 0.9999	ns
CCI ipsi vehicle *vs.* naive vehicle	> 0.9999	ns
CCI contra vehicle *vs.* naive vehicle	< 0.0001	***
CCI ipsi vehicle *vs.* CCI contra vehicle	< 0.0001	***
CCI ipsi 1 µM DAMGO *vs.* naive 1 µM DAMGO	> 0.9999	ns
CCI contra 1 µM DAMGO *vs.* naive 1 µM DAMGO	> 0.9999	ns
CCI ipsi 1 µM DAMGO *vs.* CCI contra 1 µM DAMGO	> 0.9999	ns
CCI ipsi 10 µM DAMGO *vs.* naive 10 µM DAMGO	0.298	ns
CCI contra 10 µM DAMGO *vs.* naive 10 µM DAMGO	0.1141	ns
CCI ipsi 10 µM DAMGO *vs.* CCI contra 10 µM DAMGO	> 0.9999	ns
CCI ipsi 100 µM DAMGO *vs.* naive 100 µM DAMGO	> 0.9999	ns
CCI contra 100 µM DAMGO *vs.* naive 100 µM DAMGO	> 0.9999	ns

Supplementary table 1: Test details for data shown in Figure 3.3. Effects of DAMGO on thallium uptake in DRG neurons of naïve mice and mice subjected to CCI in the absence of Kv and KATP channel blockers. (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; Kruskal-Wallis one-way ANOVA followed by Dunn's test)

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Eidesstattliche Versicherung

Ich, Philip Stötzner, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Opioids in Neuropathic Pain - The Role of Potassium Channels in Peripheral Sensory Neurons“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE - *www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.

Berlin, Philip Stötzner

Lebenslauf

Der Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version der Arbeit nicht veröffentlicht.

Publikationsliste

Blaum WE, Jarczweski A, Balzer F, **Stötzner P**, Ahlers, O. (2013): *Towards Web 3.0: taxonomies and ontologies for medical education - a systematic review*. In: GMS Z Med Ausbild. 2013;30(1):Doc13. doi: 10.3205/zma000856

Danksagung / Acknowledgment

I would like to thank my supervisor, Prof. Halina Machelska, for giving me the opportunity to realize this project in her lab, for her endless time and patience discussing the matter and for all her support along the way.

Vielen herzlichen Dank, Viola Spahn, für Deine ausdauernde, verbindliche Begleitung und die vielen Stunden geduldigen Durchsprechens von Experimenten, Ergebnissen und Konzepten.

I cannot thank you enough, Özgür Celik, for your companionship and moral support during the ups and downs of this project. Thank you for tea and scientific as well as unscientific conversations!

Grazie, Giovanna Del Vecchio, per i tuoi consigli, per pazientemente ascoltare e rispondere a tutti i miei molti problemi piccoli e grandi. Grazie per la tua sincerità scientifica intransigente.

Thank you, Dominika Labuz and Raissa Stayszyk, for managing animal matters.

Danke, Barbara Trampenau und Nicole Vogel, für eure Hilfe mit den Zellkulturen.

Außerdem gilt Henrik Alle Dank für trouble shooting bei den patch clamp Experimenten und Paul Heppenstall für Beratung bei der Datenauswertung derselben.