Peripheral opioid receptors and inhibition of sensory neuron excitability following nerve injury

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Abbreviations

ANOVA	analysis of variance
AM	A-mechanonociceptors
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
BNI	binaltorphimine
BSA	bovine serum albumin
Ca	calcium
CCI	chronic constriction injury
CGRP	calcitonin gene-related peptide
C-M	C-mechanonociceptors
C-MC	C-mechanocold
C-MH	C-mechanoheat
C-MHC	C-mechanoheatcold
CNS	central nervous system
contra	contralateral side of the injury
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2
DAB	3,3'-Diaminobenzidine
DAMGO	D-Ala^2, N-MePhe^4, Gly-ol-enkephalin
DADLE	D-Ala^2, D-Leu^5-enkephalin
DPDPE	D-Pen^2,d-Pen^5-enkephalin
DTLET	D-Thr^2-Leu^5-enkephalin-Thr
DRG	dorsal root ganglia
END	β- endorphin
ENK	enkephalin
FITC	Fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
HTMR	high-threshold mechanoreceptors
IB4	isolectin B4
IHC	immunohistochemistry

Ipsi	ipsilateral side of the injury
L	lumbar DRG
m	meter
MIA	mechanically insensitive afferents
mRNA	messenger ribonucleic acid
Na_v	voltage-gated sodium channels
NF	neurofilaments
NFG	nerve growth factor
NMDA	*N*-methyl *D*-aspartate glutamate
PBS	phosphate buffered saline
PFA	paraformaldehyde
PGP 9.5	protein gene product 9.5
PNS	peripheral nervous system
PSL	partial sciatic nerve ligation
RA	rapidly-adapting
RAM	rapidly-adapting mechanoreceptors
RM	repeated
Runx1	runt related transcription factor 1
SA	slowly-adapting
SAM	slowly-adapting mechanoreceptors
s	seconds
SEM	standard error of mean
SIF	synthetic interstitial fluid
SNL	spinal nerve ligation
SP	substance P
TNFα	tumour necrosis factor alpha
TrkA	tyrosine kinase receptor, type 1
TRP	transient receptor potential
TTX	tetrododoxin

1 Introduction

This study investigates the expression pattern of opioid receptors as well as primary sensory nerve fiber responses upon their activation in a peripheral neuropathic pain condition. For this purpose, a common animal model of neuropathic pain, the chronic constriction injury (CCI), was first used to investigate the expression of mu- (μ) and delta- (δ) receptors on different sites of peripheral sensory neurons and, secondly, to evaluate sensory nerve fiber action potentials (discharges) during neuropathic pain and upon activation of mu-receptors. A special emphasis was put on sensory nerve fiber subtypes responsible for the transmission of painful (noxious) stimuli. Hence, the introduction is divided into three main parts: the first part describes primary sensory neurons of the somatosensory system, the second one deals with pain in general and chronic, pathological pain in more detail, and the last part introduces opioids and opioid receptors and characterizes their expression and functional relevance in pain control.

1.1. Somatic sensation

The somatic sensory system is part of the peripheral nervous system and comprises at least four senses: touch, temperature, body position and pain. It includes all nerves innervating the skin, the joints or the muscles that are under voluntary control (Bear, 2001). In contrast, neurons innervating internal organs, blood vessels and glands are part of the autonomic or visceral nervous system.

In contrast to other sensory systems like vision, audition, olfaction or taste, the somatic sensory receptors are distributed throughout the body rather then being concentrated at small specialized locations. The main sensory organ is the skin and most of the sensory receptors are mechanoreceptors. Neurons bringing information from the somatic sensory system receptors to the spinal cord or the brain stem are called primary afferent neurons. The cell body resides in the trigeminal ganglia or dorsal root ganglia (DRG) of the spinal cord and is surrounded externally by satellite glial cells, which are responsible for regulation and maintenance of the microenvironment surrounding the neuronal perikarya. The DRGs are segmentally arranged adjacent to the spinal cord between the vertebrae. Primary afferent neurons belong to a subclass of bipolar cells with a so-called pseudo-unipolar morphology where one major process bifurcates into a peripheral and a central axon entering the peripheral tissue and the central nervous system (CNS), respectively (Woolf and Ma, 2007).

The afferent axons are enveloped by Schwann cells, which produce the myelin sheaths (myelinated fibres), or by Schwann cell cytoplasmic processes (unmyelinated fibres). The conduction velocity of sensory fibers depends on the diameter and myelination of the axon: the larger the diameter and myelin thickness, the faster the conduction velocity (Waxman, 1980). Accordingly, somatosensory neurons are divided into Aα, Aβ, Aδ and C fibers. Aα and Aβ fibers have the largest diameter, have thickly myelinated axons and are therefore fast conducting fibers (in mice at a speed of over 10 m/sec). Aδ fibres are thinly myelinated and have a medium diameter axon, propagating action potentials between 1 and 10 m/sec, whereas C fibres possess axons which are small in diameter, unmyelinated and conduct action potentials with a speed of under 1 m/sec in mice (Koltzenburg et al., 1997). The above indicated conduction velocities apply only to mice; the values (especially of Aα, Aβ, and Aδ fibers) vary between species. Aα fibers are proprioceptors of skeletal muscles and are therefore absent in the skin. In contrast, Aβ fibers innervate mechanoreceptors, like Merkel cells, Pacinian corpuscles and hair follicles in the skin and detect texture, vibration as well as light touch. The majority of both sub-types are responsible for the transduction of innocuous stimuli, whereas around 70% of Aδ fibers and 90% of C fibers likely detect noxious stimuli (Fang et al., 2005; Julius and Basbaum, 2001). They are called nociceptors (from the Latin word "*nocere*", which means "to hurt").

The response characteristics of mammalian sensory afferent terminals to stimulation have been studied extensively since the 1950ies using *in vivo* preparations of cutaneous sensory nerves from cats, dogs and other species. Nociceptors have been studied for the first time in 1969 by Bessou and Pearl (Bessou and Perl, 1969). The first *in vitro* preparations of sensory nerves and their attached skin appeared in the late 1950s and were conducted mainly in invertebrate animals, for example in frogs (Catton, 1958). Mammalian models appeared later and proved to be more difficult to perform. In the 1980ies, Reeh investigated extracellular recordings from single afferent units with the help of an *in vitro* preparation of rat and guinea pig saphenous nerves (Reeh, 1986). This technique is known as the *in vitro* skin nerve preparation. It enables extracellular recordings of action potentials propagating from the receptive fields of single sensory nerve endings in the skin to the spinal cord. Moreover, the *in vitro* preparation offers the advantage of pharmacological manipulation of isolated sensory receptive fields and well-controlled stimulation of the receptive fields.

In 1997, Koltzenburg and Lewin adapted this technique to study the sensory neurons of mouse saphenous nerves (Koltzenburg et al., 1997). The majority of sensory neurons can be activated by *in vitro* mechanical stimulation (Kress et al., 1992) and importantly, *in vitro*

properties of low threshold mechanoreceptors and mechanonociceptors are generally comparable to *in vivo* recordings in the same and other species like human, monkey and cat (Koltzenburg et al., 1997; Lewin and McMahon, 1991; Maurer et al., 2007; Perl, 1968).

1.1.1. Different subtypes of somatic sensory neurons

Besides the classical parameters that functionally characterize primary sensory afferents as Aβ, Aδ and C fibers, they vary in persistence of their responses to long-lasting mechanical stimulation. Some respond quickly at first but stop firing although the stimulation continues. Others produce more sustained action potentials during a long stimulus. The former ones are called rapidly adapting, the latter ones slowly adapting. Accordingly, Aβ and Aδ fibers can be subdivided into two broad types: (i) rapidly adapting mechanoreceptors that respond only to movement and (ii) slowly adapting mechanoreceptors that respond to both, movement and static indentation (Fig. 1.1).

RAM and SAM

Rapidly adapting mechanoreceptors (RAM) of the Aβ type innervate velocity-detecting receptors like Pacinian or Meissner corpuscles in glabrous skin and are mainly connected to hair follicles in hairy skin (Iggo, 1985). The slowly adapting mechanoreceptors (SAM) of the Aβ type can be classified as SA-I and SA-II mechanoreceptors, which both respond to vertical displacement of the skin with dynamic and static components. These innervate Merkel cells and Ruffini endings, respectively (Iggo, 1985).

D-hairs and A-mechanonociceptors

There are two major subgroups of Aδ fibers: low-threshold rapidly adapting D-hair receptors, and slowly-adapting A-mechanonociceptors (AM) or high-threshold mechanoreceptors (HTMR). D-hairs were first investigated in the cat and named after the hair type, whose movement most likely activated them. Down or sinus hairs are the shortest, finest and most numerous hairs of the coat. The conduction velocities are in the Aδ range and D-hair units are the only kind of hair follicle units found in this range of conduction velocities (Brown and Iggo, 1967). Interestingly, D-hairs are reckoned to be, by far, the most sensitive mechanoreceptors in the skin (Koltzenburg et al., 1997; Lewin and McMahon, 1991; Woodbury et al., 2001).

AM fibers have free nerve endings in the skin. They typically have Aδ-fiber conduction velocities although receptors with characteristics of AM neurons are also encountered with conduction velocities in the Aβ range. Still, purely afferent nerves are supposed to be dominated by low threshold mechanoreceptive Aβ (Djouhri and Lawson, 2004). AMs are effectively excited only by strong mechanical stimulation of the skin (Birder and Perl, 1994), and the evoked response frequency is graded according to intensity. AM fibers can be sub-divided into those having an additional clear response to thermal stimuli, like heat or cold (Cain et al., 2001).

C fibers

C fibers comprise the largest group of primary afferent neurons innervating the skin (60 to 70%). All C fibers have free nerve endings in the skin and respond to static indentation. They are mainly characterized further by the nature of the activating stimulus. Previous studies have revealed that most high-threshold C-fibers in the cat, rat and monkey are so called polymodal receptors and respond to heat, intense mechanical stimulation and irritant chemicals such as acids (Bessou and Perl, 1969; Birder and Perl, 1994). A minor group of C fibers can be only excited mechanically and is not activated by, or inconsistently responsive to any of the other stimuli (Bessou and Perl, 1969). Starting in the early 1980ies a straightforward classification scheme was established to describe the sensory receptors according to the range of mechanical and thermal stimuli that activated them. Thus, unimodal C fiber nociceptors are C-mechanonociceptors (C-M), whereas polymodal C fiber nociceptors can be classified as C-mechanoheat (C-MH), C-mechanocold (C-MC), or C-mechanoheatcold (C-MHC) (Fleischer et al., 1983; Kress et al., 1992). There exists also a small group of C fibers that exhibits a low mechanical threshold. Early results obtained by the use of stimuli graded from innocuous to noxious emphasized that high-threshold C fiber signal stimuli that can be threatening and damaging, whereas this is not possible by the low-threshold units (Bessou and Perl, 1969). In contrast, Meyer and Campbell identified a second substantial group of C-fibers in monkeys that displays little or no mechanosensitivity under physiological conditions (Meyer et al., 1991). These mechanically insensitive afferents (MIA) have sometimes been called "silent" or "sleeping" nociceptors (McMahon and Koltzenburg, 1990), since they have been shown to become responsive to mechanical stimulation following local inflammatory reactions.
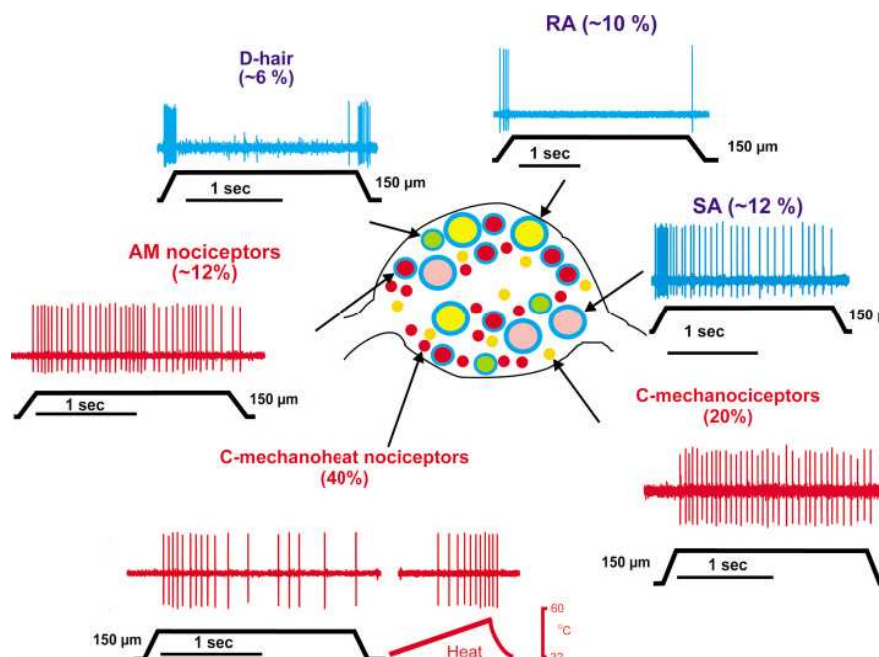
Figure 1.1: **Typical subtypes of mechanoreceptors**. The differentiation is based on threshold (in blue are low-threshold mechanoreceptors, responding robustly to the ramp phase of the stimulus; in red are all nociceptive, high-threshold mechanoreceptors responding primarily to the static phase of the stimulus), discharge rates (typical response properties of mouse mechanoreceptors from the saphenous nerve to a standardized 2 s mechanical stimulation of 150 μm are shown and differentiate rapidly (RA(M) and D-hair units)-and slowly adapting (SA(M) and AM units) mechanoreceptors), and myelination (cell bodies of the dorsal root ganglia show the approximate cell size and myelination state (myelin is indicated in blue). The approximate incidence (% of total cutaneous sensory neurons) is indicated next to the name. Adapted from Lewin and Moshourab, 2004.

1.1.2. Nociceptors

Nociceptors are unique among sensory receptors, because of their ability to detect a wide range of stimuli modalities and by the possibility to modulate their receptive properties to a high extend (Basbaum, 1999). Nociceptive Aδ and C fibers terminate in the superficial lamina of the dorsal horn (lamina I and II) as well as in the reticulated region of lamina V, in contrast to large fibres of the Aβ group that terminate within deeper lamina (lamina III and IV) (Wilson and Kitchener, 1996). Myelinated Aδ fiber nociceptors respond like the unmyelinated C fiber nociceptors, but do it more robustly and mostly have higher discharge frequencies. The two fiber types are made responsible for distinct pain sensations that are described as first and second pain: Aδ nociceptors modulate the first, sharp and fast pain, whereas C fibers transmit signals that trigger the second, dull, longer-lasting and more diffuse pain (Hunt and Mantyh, 2001; Julius and Basbaum, 2001).

Another form of classification is based on different expression patterns. In the adult DRG, histochemical studies have revealed two main classes of unmyelinated C fibers. All

newly formed embryonic nociceptors express tyrosine kinase receptors, type 1 (TrkA). However, one group of cells maintains TrkA expression and starts to express calcitonin gene-related peptide (CGRP), substance P (SP) and other peptides and is therefore called peptidergic nociceptors. The peptidergic nociceptors terminate in the lamina I and in the outer lamina II of the spinal cord. The second group switches off TrkA, continues the expression of runt related transcription factor 1 (Runx1), and starts to express a receptor tyrosine kinase named Ret. They are called nonpeptidergic nociceptors and terminate in the inner lamina II of the spinal cord (Coimbra et al., 1974; Silverman and Kruger, 1990).

There are different possibilities to biochemically identify small-diameter neurons or nociceptors. Some neurochemical markers are able to distinguish between A and C fibers. A good example is the neurofilament 200 (NF200) that is limited to neurons with A-fibers (Lawson and Waddell, 1991). Furthermore, nonpeptidergic and peptidergic C-fiber neurons can be differentiated by binding α-D-galactosyl-binding lectin B4 (IB4) or expressing SP and/or CGRP (Hunt and Mantyh, 2001). IB4 and SP are quite specific indicators for C-fiber neurons in rat and mouse (McCarthy and Lawson, 1989; Wang et al., 1994), whereas CGRP is expressed by all types of peptidergic primary afferents (McCarthy and Lawson, 1990). These markers have been useful in histochemical studies on DRG neurons in naïve animals, but it was not clear whether they also distinguish between fiber types in animal models of pain. Recently, Ruscheweyh et al. used mice that had received a chronic constriction injury (CCI) of the sciatic nerve (see chapter 1.2.3). The group could demonstrate that the same markers that are used in naïve animals selectively stain A or C, and peptidergic or nonpeptidergic nerve fibers from neuropathic animals (Ruscheweyh et al., 2007). Moreover, whereas clearly detectable levels of SP and CGRP were found in high-threshold C fiber somata, only weak or undetectable levels could be demonstrated in low threshold C fibers (Lawson et al., 1997), indicating that both markers are expressed by nociceptive C fibers. The predominant excitatory neurotransmitter in all nociceptors is glutamate (Julius and Basbaum, 2001).

1.2. Pain

Nociception and pain are vital to life. Nociception is the sensory process providing signals that trigger pain, whereas pain itself is the actual perception of a normally irritating sensation. As pain is a strongly subjective feeling that depends on an individual's personal experience and emotional status, nociception does not necessarily lead to the perception of

pain. The Taxonomy Committee of the International Association for the Study of Pain has defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Loeser and Treede, 2008). Actual and potential tissue damage provides the basis of the vital alert function of pain: the unpleasant feeling elicits withdrawal reflexes and thereby saves tissues from subsequent harm (for example, the hand is removed from a hot stove before it gets burned). The question, whether pain has distinct processing machineries or simply results from an excessive stimulation of other sensory processing units, is a very old one. Already in 1903, Charles Sherington described noxious stimuli and their responding nerve endings, which he termed nociceptors (Sherrington, 1903). In contrast, in the 1960ies and 70ies, Wall and Melzak belonged to a group of scientists, who argued that the separation of cutaneous modalities occurs at the first at the spinal cord level or even not until supraspinal processing steps (Melzack and Wall, 1962). Nowadays, the existence of specialized nerve fibers that are responsible for the transmission of painful stimuli is generally accepted (see 1.1.2). People with a congenital absence of pain, for example, report pain as a separate sensation, because the patients are still capable of discriminating normal touch sensation. So far, the syndrome's cause was either a loss of exclusively nociceptive neurons or a loss of function mutation affecting only nociceptive neurons (Cox et al., 2006; Goldberg et al., 2007; Verpoorten et al., 2006). Another fact that assigns pain to a distinct sensation is its ascending pathway. Nociception is conveyed to the thalamus through contralateral anterolateral pathways and in contrast to the touch pathway, both, direct and indirect paths to the thalamus exist. In general, secondary spinal neurons project to the contralateral side of the spinal cord and ascend in the anterolateral white matter, terminate in the thalamus, where third-order neurons project to the somatosensory cortex, cingulate gyrus (part of limbic system) and insular cortex. The most prominent ascending nociceptive pathway is called the spinothalamic tract (Basbaum and Jessell, 2000). The pathways transmitting touch are entirely distinct from those transmitting pain and temperature. While information about pain and temperature ascends contralaterally, information about touch ascends to the medulla through the ipsilateral dorsal horn, along the dorsal column-medial lemniscal pathway.

Pathological pain includes inflammatory and neuropathic pain. Injuries to the nervous system can result in a substantial functional loss and impaired sensory and motor functions as well as secondary problems, such as neuropathic pain (Navarro, 2009). Neuropathic pain normally persists long after the initiating event has healed (Woolf and Salter, 2000) and importantly, looses its vital function in the way that the pain neither protects nor supports

healing or repair (Costigan et al., 2009). In contrast, the pain syndromes result from a malfunction of the somatosensory system itself and neuropathic pain can be considered as a disease on its own.

1.2.1. Neuropathic pain

The Taxonomy Committee of the International Association for the Study of Pain describes neuropathic pain as: "Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (Loeser and Treede, 2008). Persistent neuropathic pain normally involves the establishment of a stimulus-evoked sensitization or hypersensitivity (hyperalgesia and allodynia) and spontaneous pain (Woolf and Mannion, 1999). Allodynia is characterized as "pain in response to a normally non-noxious stimulus", whereas hyperalgesia is described as "increased pain sensitivity" in response to normally noxious stimuli (Loeser and Treede, 2008). Both symptoms can occur in response to thermal or mechanical stimulation and can be measured in animals, for example, by measuring the latency of hind paw withdrawal from a radiant heat source or by measuring the threshold of paw withdrawal to calibrated von Frey hairs of increasing forces, respectively. Spontaneous pain, in contrast, describes pain that lacks any identifiable, inducing stimulus. Different mechanisms have been elucidated that are thought to be responsible for the behavioural symptoms encountered in neuropathic pain patients and animal models. These mechanisms can be broadly distinguished by the location of neurons that are affected. A nerve injury on a peripheral sensory nerve could result in hypersensitivity of primary sensory afferents (peripheral sensitization) and/ or of second-order or interneurons in the spinal cord or brain (central sensitization).

Peripheral sensitization most commonly involves neuro-immune interactions and therefore results from inflammation-associated changes in the chemical environment of the nerve fiber. Following injury to a peripheral nerve, degeneration of axons in the distal segment, a process known as Wallerian degeneration, is initiated (Koeppen, 2004). In turn, primary nociceptive afferents release neuropeptides like SP and CGRP at the level of the spinal cord and in the periphery (Jang et al., 2004). This is thought to contribute to the induction and partly to the maintenance phase of neuropathic pain by facilitating and promoting the release of inflammatory factors from neighbouring non-neuronal cells, a phenomenon called neurogenic inflammation (Julius and Basbaum, 2001). Injection of antagonists of the SP and CGRP receptors (neurokinin1 and CGRP1, respectively) at the level of the spinal cord (Cahill and Coderre, 2002) or in the peripheral tissue (Jang et al., 2004) attenuated certain neuropathic pain symptoms. Nerve injury therefore leads to an upregulation

and release of proinflammatory substances from resident Schwann cells, mast cells, endothelial cells and fibroblasts (Myers et al., 2006). One of the released cytokines is tumour necrosis factor alpha (TNFα), whose main function seems to be the recruitment of macrophages from the blood (Liefner et al., 2000). They, in turn, dominate the process of degeneration and phagocytosis of myelin debris initially started by resting Schwann cells. At later stages, Schwann cells play a crucial role in neuroregeneration, as they possess a basal lamina that degenerates very slowly and therefore serves as a conduit for nerve fiber regrowth (Ide et al., 1983). The release of substances from the inflammatory soup, including TNFα, adenosine triphosphate (ATP), bradykinin, histamine and neurotrophic factors like nerve growth factor (NGF), renders nociceptor terminals exposable to these products. Activation of their receptors on nociceptors induces intracellular signal transduction pathways that increase the production, transport and membrane insertion of transducer channels and voltage-gated ion channels (Costigan et al., 2009). NGF, for example, binds to its receptor TrkA on peptidergic C fiber nociceptors and activates downstream signalling pathways that result in functional potentiation and increased expression of pronociceptive proteins (Basbaum et al., 2009). Among others, the function of heat-activated transient receptor potential V1 (TRPV1) channels was recently shown to be upregulated in IB4-positive primary sensory neurons after spinal nerve injury and it was suggested that this effect largely contributes to the behavioural heat hypersensitivity (Vilceanu et al., 2009).

Voltage-gated sodium channels (Na_v) are the major class of ion channels thought to contribute to the increased excitability of neurons in neuropathic pain. The tetrododoxin (TTX)-resistant $Na_v1.8$., for example, is predominantly expressed in nociceptors and although TTX-resistant current densities were reduced following axotomy (Dib-Hajj et al., 1996) or spared nerve injury (Berta et al., 2008), the knockout model of Nav1.8 (Roza et al., 2003) or its knockdown by antisense oligodeoxynucleotide (Joshi et al., 2006) prevented the hyperalgesia and allodynia following peripheral nerve injury. Moreover, a de novo expression of a TTX-sensitive Na^+ channel (Nav1.3), has been reported after peripheral nerve injury (Waxman et al., 1994). Nociceptive transmission also largely depends on various types of voltage-gated Ca^{2+} channels that regulate synaptic transmission. High-voltage-gated calcium channels include three subfamilies, designed as L-type, N-, P/Q-, and R-type and T-type, all expressed in neuronal tissue (Gribkoff, 2006). N-type Ca^{2+} channels, for example, were upregulated in DRGs after nerve injury and if deleted or if blocked at the nerve injury site or spinal cord, reduced neuropathic pain symptoms in mice and rats (Basbaum et al., 2009; Matthews and Dickenson, 2001; Saegusa et al., 2001). Also, after nerve injury, T-type

channel current density was increased in small DRG neurons (Jagodic et al., 2008) and blocking of an isoform of T-type channels reduced tactile and thermal hypersensitivities (Dogrul et al., 2003). Together, the accumulation or rearrangements of voltage-gated ion channels in the axon and DRGs following nerve injury have been implicated in the establishment of neuropathic pain and specifically in the heightened excitability of nociceptors.

The CNS reacts to changes in the activity of primary afferent neurons and to the release of neuropeptides, neurotrophic factors and inflammatory mediators at the level of the spinal cord. Similar to the periphery, nerve injury activates spinal cord glia and causes these cells to enhance pain by releasing proinflammatory cytokines, glutamate and brain-derived neurotrophic factor (BDNF) (Wieseler-Frank et al., 2005). Although it has been shown that SP mRNA decreases in the DRG after axotomy, peripheral nerve injury induces phenotypic shifts in large diameter A-fibers in a way that they start to express SP and BDNF (Noguchi et al., 1995; Zhou et al., 1999), which may enable these fibers to evoke central sensitization. In addition, there is a central reorganization of large diameter A-fibers, which start to sprout into lamina II of the spinal dorsal horn where C-fibers normally terminate and make functional synaptic contacts (Woolf et al., 1992). In this way, low threshold Aβ fibers are thought to begin to transmit pain after nerve lesion. Another group of neurons affected are inhibitory dorsal horn interneurons that modulate afferent input by synapsing with the central terminals of primary sensors neurons. Among others, a loss of inhibitory gamma-aminobutyric acid (GABA) interneurons has been reported after nerve injury (Scholz et al., 2005). In the setting of nerve injury, repetitive firing and the following increased release of transmitters (glutamate and SP) from nociceptive primary afferent neurons can sufficiently depolarize postsynaptic neurons to activate normally quiescent N-methyl D-aspartate glutamate (NMDA) channels (Basbaum et al., 2009). This in turn can strengthen synaptic connections and exacerbate responses to noxious stimuli.

1.2.2. Primary sensory neurons and neuropathic pain

From a therapeutic perspective, primary afferent neurons are of particular interest, because they are the initial generators of action potentials relaying nociceptive information towards the spinal cord and the brain. Thus, if one could inhibit the sensitization and/or excitation of peripheral sensory neurons, subsequent central events such as sensitization and plasticity may be prevented or counteracted. Injuries to a peripheral nerve and their accompanying neuro-immune interactions (see 1.2.1) are thought to lead to a heightened

excitability and spontaneous, ectopic firing of primary afferent neurons, including nociceptors. As primary afferents are designed to initiate activity only at their peripheral terminals, any action potential originating from the axon or cell body represents pathological ectopic ("occurring in an abnormal position or place") firing (Woolf and Ma, 2007). Various studies reported substantial ectopic activity in primary sensory neurons following nerve injury and the spontaneous activity was generated at multiple sites, including the neuroma site (site of injury with aborted axon growth), the cell body of injured DRG neurons (Amir et al., 2005) and neighbouring intact afferents (Wu et al., 2001). Moreover, evidence suggests that behaviourally assessed spontaneous pain is based on spontaneous firing of nociceptive neurons (Djouhri et al., 2006).

However, if and how primary afferent neurons display an increased excitability to thermal, chemical or mechanical stimulation of their peripheral receptors after nerve injury, has only been partly investigated. Of the different modalities, a hypersensitivity to mechanical stimulation has been reported by numerous behavioural studies in animals investigating the consequences of peripheral nerve injuries like the CCI of the sciatic nerve (Bennett and Xie, 1988; Labuz et al., 2009; Truong et al., 2003). Moreover, touch-evoked pain is a common clinical phenomenon of peripheral neuropathic pain conditions and it can be particularly distressing to patients, as tactile stimulation and therefore pain is inevitable (Baron, 2006; Costigan et al., 2009).

Elucidating the underlying neuronal mechanisms of mechanical hypersensitivity following nerve injury requires the examination of (1) whether the sensitization to mechanical stimuli is reflected in alterations of primary afferent neurons and (2) which parameters encode it. With respect to the latter, the mean number of action potentials (discharges) of nociceptors per a constant mechanical stimulation is a parameter that has been shown to correlate well with the strength of the mechanical stimulus and the perceived pain magnitude (Handwerker et al., 1987b; Koltzenburg and Handwerker, 1994). This suggests that elevation in the discharge rates is a possible sign of a hypersensitive state of primary afferent nociceptors. Moreover, a decrease in the thresholds to thermal or mechanical stimulation of nociceptors is considered as an indication of their sensitization. Consistently, sensitization to heat in form of a drop in the mean heat threshold of nociceptors and/or an increased discharge rate to heat stimulation frequently appeared following repetitive noxious thermal stimulation of healthy skin or heat stimulation under inflammatory and neuropathic conditions (Beitel and Dubner, 1976; Campbell et al., 1979; Fitzgerald, 1979; Jankowski et al., 2009; Kirchhoff et al., 1990; Kocher et al., 1987; Koltzenburg et al., 1999; LaMotte et al., 1992; Randich et al., 1997; Shim

et al., 2005). However, controversial data about sensitization to mechanical stimulation exist. Under inflammatory conditions, some studies have reported an increase in the total discharges evoked during a constant mechanical stimulus for mechanosensitive C fibers or a decrease in the median von Frey threshold of C fibers (Andrew and Greenspan, 1999; Randich et al., 1997; Wenk et al., 2006). In contrast, several other studies did not observe sensitization of cutaneous nociceptors to mechanical stimuli in such condition (Banik and Brennan, 2004, 2008; Handwerker et al., 1987a; Kirchhoff et al., 1990; LaMotte et al., 1992; Milenkovic et al., 2008; Schlegel et al., 2004).

Of note, unlike inflammation, very few studies addressed the effects of mechanical stimulation on cutaneous nociceptor responses following traumatic nerve damage. Here, an increase in the discharge rate and a decrease in the mechanical thresholds of Aδ and C fibers to a single suprathreshold mechanical stimulus following a spinal nerve ligation (see 1.2.3) (Shim et al., 2005) or decreased thresholds of Aδ, but not C nociceptors after nerve transsection (Jankowski et al., 2009) have been demonstrated. In contrast, a slightly higher mechanical threshold of C fibers after a CCI in rodents has been reported (Koltzenburg et al., 1994).

1.2.3. The CCI as an animal model of neuropathic pain

A great advance in the study of neuropathic pain came from the discovery that the placement of loose chromic gut ligatures on the sciatic nerve of the rat resulted in a phenotypic behaviour similar to the one in neuropathic pain patients (Bennett and Xie, 1988). The rats had lowered thresholds to heat, cooling, and mechanical stimuli (indicating hypersensitivity) and showed some symptoms of spontaneous pain for a period of more than 2 months (Attal et al., 1990; Bennett and Xie, 1988). Moreover, the CCI simulates the symptoms of chronic nerve compression, and importantly, many painful neuropathies in the clinical situation rather involve a partial nerve damage then total transection of the nerve (Bennett, 1993). The loose constriction of the nerve leads to intraneural oedema, a focal ischemia, and an axonal (Wallerian) degeneration. The chromic gut used induces a local inflammatory reaction. This inflammation is accompanied by an immune reaction including invasion of immune cells (Kleinschnitz et al., 2006) also found in a CCI in mice ligated with silk sutures (Labuz et al., 2009). Besides the CCI, common animal models of neuropathic pain include the partial sciatic nerve ligation (PSL) and the spinal nerve ligation (SNL) (Fig. 1.2). The PSL, like the CCI, involves a partial denervation of the sciatic nerve made by one tight ligature around up to 1/2 of the diameter of the sciatic nerve, which affects axons of all sizes

(Bennett et al., 2003; Seltzer et al., 1990) and is often referred to as a tight ligation. The SNL involves two ligatures at the level of the spinal nerves (around L5 and L6) and produces a partial denervation of the sciatic nerve similar to the PSL (Bennett et al., 2003; Kim and Chung, 1992). An important feature of partial nerve injury models such as CCI, PSL and SNL is the coexistence of axotomized and degenerating axons of primary afferents with spared functioning axons in the partially denervated skin.
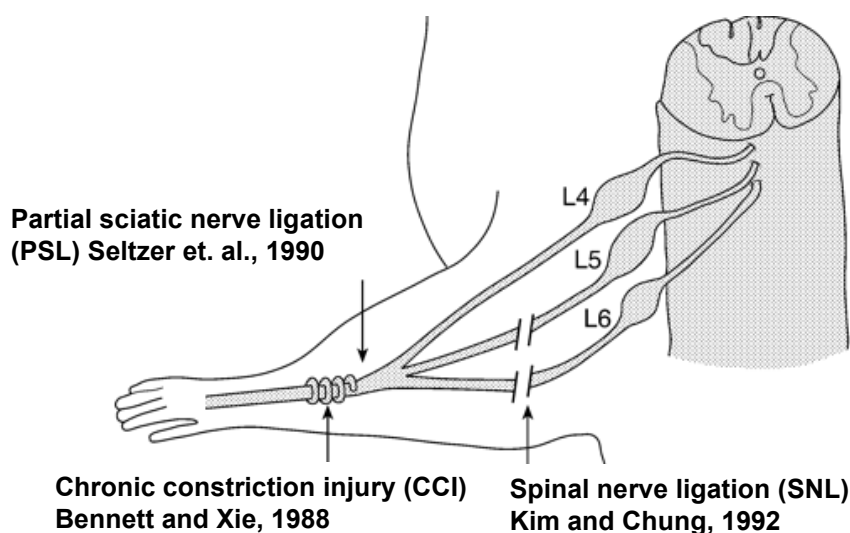
Figure 1.2: Common animal models of neuropathic pain in the sciatic nerve. The scheme includes the CCI, indicated with 3 ligatures only (used for mice). Modified after Bridges et al., 2001.

1.2.4. Treatment of neuropathic pain

Neuropathic pain emerged as a generic term for diseases whose complex pathophysiology are still not clearly understood (Allen, 2008; Sindrup and Jensen, 1999). It may have multiple origins including poststroke and spinal cord injury pain of central nervous system origin. However, neuropathic pain resulting from diseases or injuries to a peripheral nerve is even more relevant in the clinic: Current studies state that neuropathic pain affects 1–3% of the developed world population and that diabetic neuropathy following diabetes in patients is the most common cause of neuropathy in the Western world. Moreover, over 60% of patients aged over 60 years develop postherpetic neuralgia after acute herpes zoster (Allen, 2008). Additional causes of peripheral origin include HIV sensory neuropathy, cancer, phantom limb pain and posttraumatic nerve injuries (Backonja and Serra, 2004). Pharmacotherapy of neuropathic pain employs tricyclic antidepressants or anticonvulsants, but effective analgesia is achieved in fewer than 50% of patients and both are associated with

sub-optimal side effects (Bridges et al., 2001; Sindrup and Jensen, 1999). The use of opioids for chronic pain in general and neuropathic pain in particular has increased dramatically over the last decade. Yet, the belief that neuropathic pain is opioid-resistant, as well as concerns of their adverse side effects often discouraged the use of opioids for neuropathic pain.

1.3. Opioids

Opiates or opioides name a group of substances that act on opioid receptors and thereby elicit a variety of responses that are in first instance associated with positive, pain-relieving (analgesic) consequences. In earlier years a distinction was made between "opiates" being exogenous, naturally occurring alkaloids and "opioids", referring to the endogenous system of opioid peptides. Opioid is the term now used broadly to describe all compounds that work at the opioid receptors. It refers to the term opium that defines a mixture of alkaloids from the poppy seed of the poppy plant. The most famous exogenous opioid is morphine. In 1975, Hughes et al isolated and determined the amino acid sequence of two pentapeptides from brain extracts of pigs mimicking morphine effects, which were called methionine (met)-enkephalin and leucine (leu)-enkephalin (Hughes et al., 1975). Today, the endogenous opioid peptides include β-endorphin, the enkephalins, the dynorphins and the endomorphins. Endogenous opioid peptides are transcribed from the mRNA of a precursor gene: β-endorphin from the pro-opiomelanocortin (POMC) gene, the enkephalins from the pro-enkephalin (PENK) gene and the dynorphins from the prodynorphine (PDYN) gene, however, the genetic origin and precursors of the endomorphins are unknown to date (Busch-Dienstfertig and Stein, 2009).

In the peripheral nervous system (PNS), endogenous opioids are produced in reproductive, neuronal and other tissues, including immune cells (Stein et al., 2003). This finding has questioned the solely detrimental role of immune cells at a nerve injury site, whose depletion or reduction was suggested as a necessary tool to diminish pain (Liu et al., 2000; Zuo et al., 2003). Among others, granulocytes and macrophages were shown to express endogenous opioid peptides, which are released at the site of inflammation and unfold their analgesic effect by binding to opioid receptors (Labuz et al., 2006; Rittner et al., 2005). The release of endogenous opioids from immune cells can be induced by stress, but also by activation of corticotrophin-releasing factor (CRF) receptors, co-expressed with endogenous opioids under inflammatory conditions (Mousa et al., 2003).

In table 1.1, common endogenous and exogenous agonists of the three main opioid receptors, mu, delta and kappa (see 1.4) are listed. However, a cross-reactivity between all three receptors and opioid peptides are immanent; especially β-endorphin and the enkephalins bind to both, mu- and delta-receptors (Raynor et al., 1994). Of the synthetic, exogenous ligands DTLET ((D-Thr^2-Leu^5)-enkephalin-Thr), DADLE ((D-Ala^2, D-Leu^5)-enkephalin), and DPDPE ((D-Pen^2,d-Pen^5)-enkephalin) have the highest binding affinity to the δ opioid receptor, whereas DAMGO ((D-Ala^2, N-MePhe^4, Gly-ol)-enkephalin) possesses a high affinity to the mu-receptor (Standifer and Pasternak, 1997).

	mu	delta	kappa
Endogenous agonists	β- endorphin endomorphins	met-enkephalin leu-enkephalin	dynorphins
Exogenous agonists	morphine fentanyl methadone DAMGO	DPDPE DTLET DADLE	U50488H ketocyclazocine
Antagonists	naloxone CTOP	naloxone naltrindole	naloxone nor-BNI

Table 1.1: Endogenous and exogenous agonists and antagonists of the three opioid receptors. Modified after Dickenson (1991) and summarized in Minami (1995). CTOP = D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2, nor-BNI = nor-binaltorphimine.

1.4. Opioid Receptors

1.4.1. Discovery

In 1973, scientists demonstrated a direct, stereo specific binding of opioids to membrane receptors in nervous tissue of different animals (Pert and Snyder, 1973; Simon et al., 1973). Based on different pharmacological syndromes evoked by exogenously applied opioids three different opioid receptors, mu-, delta- and kappa-receptors, were proposed (Martin et al., 1976). The opioid receptor gene for delta was isolated by expression cloning in 1992 (Evans et al., 1992; Kieffer et al., 1992). Mu and kappa opioid receptor isolation followed only one year later by homology studies to the delta opioid receptor in mouse and rat brain (Chen et al., 1993; Thompson et al., 1993; Yasuda et al., 1993). Other opioid receptors were proposed, however, the identification and sequence analysis of complementary DNA

(cDNA) and the selective deletion of opioid receptor genes in mice confirmed the existence of only three genes (Kieffer and Gaveriaux-Ruff, 2002). Pharmacological subtypes may result from alternative splicing, posttranslational modifications, or receptor oligomerization.

1.4.2. Expression and signalling

Opioid receptors were first discovered in brain tissues of rats and mice. They are expressed primarily in the cortex, limbic system, and brain stem (Le Merrer et al., 2009) and show a differential distribution pattern. Mu- and kappa-receptor binding sites were found in the periaqueductal grey (PAG), locus coeruleus, substantia nigra, ventral tegmental area, raphe nuclei and nucleus tractus solitarii, while delta-receptor binding was demonstrated in the substantia nigra and nucleus tractus solitarii (Przewlocki and Przewlocka, 2001). Several brain regions including the PAG and nucleus raphe magnus (NRM) are critical sites for supraspinal pain modulation. In situ hybridization and immunohistochemistry studies have shown that in the spinal dorsal horn, opioid receptor mRNA and proteins are expressed predominantly in the superficial lamina (lamina I and II), where nociceptive C and Aδ fibers of primary afferents principally terminate (deGroot et al., 1997).

The assumption that the opioid receptors belong to the super family of G-protein coupled receptors came up very early and proved true: All three receptors have a typical seven hydrophobic transmembrane structure and upon activation, interact with certain guanine-nucleotide-binding regulatory proteins (G proteins). The transmembrane domains are connected by relatively short intracellular and extracellular loops with the amino terminus on the extracellular side and the carboxyl terminus on the intracellular side. The three opioid receptors are highly homologous. Altogether they share around 60% homology in amino acid sequence (Chaturvedi et al., 2000). That is especially true for the opioid binding site in the seven-transmembrane helical core. The extracellular domains differ more strongly between mu, delta and kappa and likely form protein gates that select the agonists or antagonists. Intracellular loops of the receptor form a large part of the receptor-G protein interface. These intracellular receptor domains are almost identical across mu, delta and kappa receptors, consistent with the fact that all three receptors interact with inhibitory G proteins of the Gi/Go type. Opioid binding to the opioid receptor induces an exchange of GDP to GTP on the Gi/o protein which dissociates from the receptor into the α and β/γ subunit (Fig. 1.3). The GTP-bound α subunit inhibits the enzyme adenylyl cyclase. This in turn leads to a decrease in intracellular cyclic adenosine monophosphat (cAMP) concentration that is responsible for numerous intracellular responses. So far, the β/γ subunit seems to mediate more and

immediate actions, which include presynaptic and postsynaptic mechanisms like the inhibition of various voltage-gated Ca^{2+} channels, as well as the activation of voltage-gated K^+ channel and G-protein-gated inwardly rectifying K^+ (GIRK) channels (Rhim and Miller, 1994; Vaughan et al., 1997; Yoshimura and North, 1983). Each of these effector systems act to reduce neuronal excitability following agonist activation of the opioid receptor.
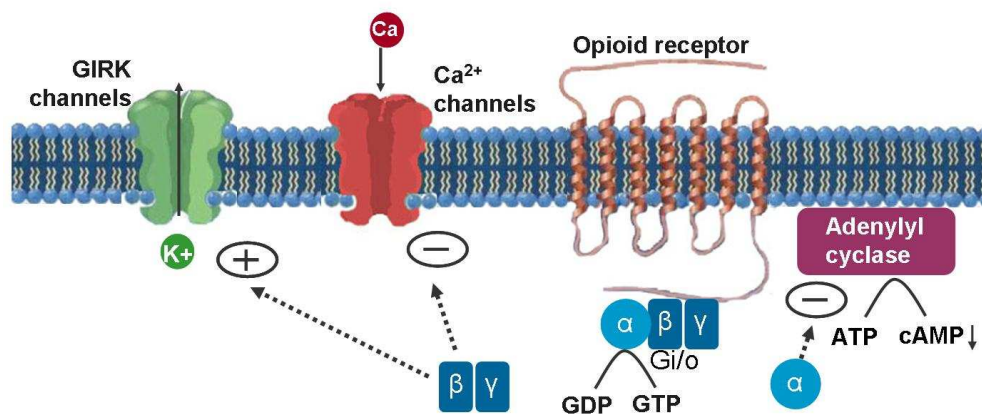
Figure 1.3: Intracellular signalling cascade of opioid receptors in the CNS. Upon activation of the opioid receptor, the G-protein α-subunit inhibits adenylyl cyclase activity and thereby reduces cAMP levels, whereas the βγ-subunit decreases Ca2+ influx and increases K+ efflux leading to reduced neuronal excitability.

1.4.3. Analgesic effect through activation of opioid receptors

One view concerning the treatment of neuropathic pain is that opioids are not effective or do not significantly reverse a hypersensitivity, in either animal models (Aley and Levine, 2002; Kontinen et al., 1998) or the clinic (Arner and Meyerson, 1988; Dworkin et al., 2007). Likewise, systemic (intraperitoneal (ip), subcutaneous (sc) or intravenous (iv)) or intrathecal morphine injections showed a lowered efficiency in reducing or preventing hypersensitivity in animal models of neuropathic pain like the SNL or spared nerve injury (SNI) (Decosterd et al., 2004; Kontinen et al., 1998; Suzuki et al., 1999). Others showed potent analgesia of supraspinal administered opioids, but reported a failure or a reduction of the efficiency of intrathecally administered morphine in alleviating mechanical or thermal hypersensitivity in the PSL or SNL model of neuropathic pain (Bian et al., 1995; Lee et al., 1995; Pertovaara and Wei, 2003; Rashid et al., 2004).

However, in contrary, studies using single-dose intravenous opioids have shown benefit in the use against neuropathic pain in the clinic (Dellemijn et al., 1998; Eisenberg et al., 2006; Rowbotham et al., 1991). Likewise, in animal studies, systemically administered

opioids showed dose-dependent antinociceptive effects to mechanical and thermal stimuli in the CCI model of neuropathic pain (Attal et al., 1991; Backonja et al., 1995; Kayser et al., 1995; Lee et al., 1994). In line with these results, many studies support the view that opioids do provide neuropathic pain relief with a sufficient dose escalation (Dickenson and Suzuki, 2005; Portenoy et al., 1990). Taken together, the efficacy of systemically administered opioids in neuropathic pain appears to be dependent on different parameters like the kind of nerve injury and the opioid dose used. However, dose-escalation after systemic administration is limited by adverse side effects that include depression of breathing, nausea, clouding of consciousness, addiction, and tolerance (Zollner and Stein, 2007). Interestingly, the activation of opioid receptors in the peripheral nervous system is devoid of centrally mediated side-effects without loosing its antinociceptive capacity. This was shown in different studies investigating inflammatory conditions (for example (Likar et al., 1997; Stein et al., 1996)). Hence, exploring the activation of peripherally expressed opioid receptor in neuropathy seems to be a more promising and less limited way in producing efficient opioid-mediated analgesia.

1.4.4. Opioid receptors in the PNS

Intense research localized all three opioid receptors also in peripheral neurons and in neuroendocrine (pituitary, adrenals), immune, and ectodermal cells (Zollner and Stein, 2007). In the peripheral nervous system, opioid receptors are mainly expressed in small- and medium-diameter DRG neurons or axons that co-express prototypical sensory neuropeptides such as SP and CGRP (Li et al., 1998; Minami et al., 1995; Mousa et al., 2007; Zhang et al., 1998a). From their production site, the DRGs, they are transported to the central and peripheral nerve terminals (Hassan et al., 1993; Li et al., 1996). Similar to the CNS, activation of peripheral opioid receptors reduces neurotransmitter release, for example the release of SP and CGRP from central and peripheral terminals of sensory neurons (Khasabova et al., 2004; Kondo et al., 2005). Peripheral opioid receptors are also coupled to Gi/o proteins that inhibit adenylyl cyclase activity and modulate ion channels (Zollner et al., 2003). Opioid receptors on DRG neurons were shown to suppress high voltage-gated Ca^{2+} currents of the N- and P/Q-type (Acosta and Lopez, 1999; Moises et al., 1994) and TTX-resistant Na^+ or nonselective cation currents (Gold and Levine, 1996; Ingram and Williams, 1994). In contrast to the central nervous system the modulation of K^+ channels by activated opioid receptors appears to be no major mechanism for the inhibition of sensory neuron functions (Akins and McCleskey, 1993). The question, whether GIRK channels are expressed and functional in the peripheral nervous system is still under debate.

Regarding the efficiency of opioids in alleviating pain in the periphery, studies have mostly investigated inflammatory conditions. Starting at the beginning of the 1990ies, some studies provided evidence that antinociceptive effects of opioids can be mediated by peripheral opioid receptors (Bartho et al., 1990; Stein et al., 1990). Besides testing the effect in animal models of pain, the activation of peripheral opioid receptors was successfully investigated in clinical studies. One of the most extensively studied applications is the intraarticular injection of morphine into inflamed knee joints (Likar et al., 1997; Stein et al., 1991). Different studies have shown that inflammation induces an increase in mu-receptor binding sites and in its protein and mRNA content within the DRGs and that this upregulation has its functional consequence in enhanced mu-receptor G-protein coupling and mu-receptor partial agonist efficiency (Mousa et al., 2001; Puehler et al., 2004; Shaqura et al., 2004; Zollner et al., 2003). Zöllner et al (2003) could show that the upregulation of mu-receptor binding sites in the DRG is due to an increase in both, the number of neurons expressing the mu- receptor and the number of mu-receptors in DRG membranes. Under inflammatory conditions, β-endorphin binding sites were largely increased within the sciatic nerve and in the ipsilateral paw tissue, suggesting an enhanced axonal transport and availability of opioid receptors (Hassan et al., 1993). Moreover, inflammatory conditions lead to a disruption of the perineurial sheath around the peripheral nerve, which could have resulted in an eased excess to opioid receptors on the sensory neurons (Antonijevic et al., 1995). All of these mechanisms are thought to contribute to an increased antinociceptive efficiency of opioids in inflamed tissue.

With respect to the effect of peripherally applied opioids on sensory primary afferent characteristics, only two studies were found that recorded single primary afferents from nerves of inflamed tissue in an in vitro skin-nerve preparation. Here, ultraviolet irradiation-induced spontaneous activity of C and Aδ fibers from saphenous nerves and the discharge rates to mechanical stimulation of C fibers from sciatic nerves after hind paw inflammation were both shown to be reduced following local application of morphine to the receptive fields (Andreev et al., 1994; Wenk et al., 2006). Both studies provided first electrophysiological evidence for functional opioid-opioid receptor interactions on the peripheral terminals of primary afferents under inflammatory or inflammatory-like conditions.

1.4.5. Peripheral opioid receptors under neuropathic pain conditions

Peripheral administrations of a mu-receptor agonist failed to reverse mechanical and thermal hypersensitivity in the PSL model of neuropathy (Aley and Levine, 2002; Rashid et al., 2004). It was suggested that this is caused by a nerve injury-induced decrease in mu-and delta-receptor protein expression in the PNS, e.g. in the DRGs or spinal nerve terminals after nerve injury, like reported after a PSL, SNL or a transsection of the sciatic nerve (deGroot et al., 1997; Goff et al., 1998; Kohno et al., 2005; Porreca et al., 1998; Rashid et al., 2004; Stone et al., 2004; Zhang et al., 1998b). In contrary, however, a CCI resulted in an upregulation in mu-receptor expression (Goff et al., 1998; Kolesnikov et al., 2007; Stevens et al., 1991; Truong et al., 2003) and a decreased or increased delta-receptor expression (Kabli and Cahill, 2007; Stevens et al., 1991; Stone et al., 2004).

Similarly, many studies provide sufficient evidence for a local, peripheral analgesic effect of opioids under neuropathic pain conditions. An intraplantar (i.pl., into the paw) injection of a mu-receptor agonist dose-dependently reversed mechanical allodynia after SNL and a majority of studies using the CCI model showed potent antinociception of peripherally acting opioid receptor agonists (Guan et al., 2008; Kabli and Cahill, 2007; Obara et al., 2009; Obara et al., 2004; Truong et al., 2003). As a lesion to a peripheral nerve is often associated with an inflammatory response, we recently could demonstrate a peripheral analgesic effect through activation of endogenous opioids. The study showed that about 30-40% of the immune cells that accumulated at the CCI site expressed opioid peptides. When these cells were stimulated by local application of CRF, an opioid peptide-mediated activation of opioid receptors resulted in abolished tactile hypersensitivity (Labuz et al., 2009). Only one report addressed the peripheral site of opioid action in patients with neuropathic pain (Azad et al., 2000), likewise reporting a significantly decreased pain in patients with a complex regional pain syndrome of the arm.

However, so far, opioid receptor expression was mostly investigated at the level of the spinal cord, less often in the DRGs (Kolesnikov et al., 2007; Rashid et al., 2004; Truong et al., 2003) and scarcely in the injured sciatic nerve. Here, a small increase in mu- or delta-receptor expression at the CCI injury site has been reported (Kabli and Cahill, 2007; Truong et al., 2003). Recent studies using Western blot analysis demonstrated an increase of mu-receptors in the hind paw skin innervated by the damaged saphenous nerve following CCI (Walczak et al., 2006). However, as Western blot analysis does not allow any conclusion on the cell types investigated, alterations in the opioid receptor content expressed at the peripheral nerve endings in the skin have not yet been investigated following a nerve injury.

Similarly, thorough analysis which considers the effect of opioid agonists on nerve-injury induced changes in the excitability of nociceptive fibers is lacking. So far, opioids have been shown to elicit modulatory actions in the spinal cord. Intrathecal administration of morphine, for example, significantly decreased the number of spontaneously generated action potentials of deep dorsal horn neurons and C fiber-evoked dorsal horn neuron responses after a SNL in rats (Suzuki et al., 1999; Suzuki and Dickenson, 2006). Additionally, the effects of morphine after a SNL have been tested on dorsal horn neuron responses to von Frey hair mechanical stimulation or to pressure to the fiber's receptive field, which were both significantly decreased after intrathecal morphine administration (Chen et al., 2005; Suzuki et al., 1999). However, alterations in the thresholds or discharge rates to mechanical stimulation of primary sensory neurons after opioid application to their receptive fields have not yet been investigated following nerve injury.

In the present study, we therefore intended to present a thorough investigation of the expression pattern of two opioid receptors, mu and delta, in the cell bodies of the DRGs, in the sensory axons at the nerve ligature site and in the sensory terminal endings in the skin following nerve injury. These sites are most relevant for an analgesic effect of opioids acting at their peripheral receptors. As the CCI of the sciatic nerve proved sensitive, first, in establishing symptoms that are very close to clinical cases of a neuropathy and, second, in responding to peripherally applied opioids, this model was chosen for investigations. Moreover, we wanted to clarify, whether the threshold to mechanical stimulation and the discharge rate to increasing mechanical stimulation of primary afferent neurons alter after a peripheral nerve injury. We additionally included the latency of response to mechanical stimulation as a possible parameter that might be influenced by a nerve injury. The primary aim of this study was to elucidate the effects of opioid receptor activation expressed on these sensory neurons on the abovementioned parameters under neuropathic pain conditions. We used a slightly modified CCI of the saphenous nerve in mice, which was tested in an in vitro skin-nerve preparation. A mu-receptor agonist, DAMGO, was administered to the receptive fields of single sensory neurons. To capture all possible alterations in primary afferent neurons, we investigated C and Aδ (AM nociceptor) fibers that are supposed to mediate noxious stimuli and expanded our analysis also onto normally non-nociceptive fiber types of Aβ (RAM and SAM) and Aδ (D-hairs) fibers.

2   Objectives

The present Ph.D. thesis focuses on opioid receptors expressed in the peripheral nervous system and the responses of primary sensory neurons upon their activation by agonists. Opioids acting on opioid receptors have been used in the treatment of pain for a few thousand years (Brownstein, 1993) and are considered the most powerful analgesics (McQuay, 1999). However, in the treatment of neuropathic pain, the literature reveals discrepancies with respect to opioid efficiency. Among current opinions, opioids are thought to be less potent in patients and in animal models of neuropathic pain compared to e.g. inflammatory pain conditions, indicating a need for increasing the dose. This option is restrained by severe side effects that appear alongside opioid administration. Common examples are addiction, tolerance, nausea or depression of breathing. However, most of the side effects are mediated by activating opioid receptors in the CNS. In contrast, the activation of opioid receptors expressed on neurons in the PNS lacks the establishment of centrally mediated side effects. Therefore, this study was designed to explore their expression and activation in a neuropathic pain model.

We decided on a common animal model of neuropathic pain, the CCI. This model simulates the symptoms of partial nerve damage, which better mirrors painful neuropathies in the clinical situation then e.g. a complete transection of the nerve (Bennett, 1993). Accordingly, the CCI preserves spared functioning axons in the partially denervated skin. That allowed us to investigate the response behaviour from nerve fibers that could be stimulated on their receptive fields, representing the natural (in situ) side of perception. For this purpose, we used an in vitro skin-nerve preparation enabling us to identify and record from singly sensory afferents. We decided to concentrate on mechanical stimulation, because the majority of sensory nerve endings identified in the skin of mammals can be driven by mechanical stimulation (Kress et al., 1992) and mechanical hypersensitivity represents one of the most frequently encountered symptoms of neuropathy (Baron, 2006; Labuz et al., 2009). To test the neuronal response upon activation of opioid receptors expressed on peripheral sensory nerve terminals, we chose the mu-receptor agonist DAMGO. Compared to other mu-receptor agonists like morphine or endomorphin, intraplantar injected DAMGO resulted in the most potent effect in behavioural studies (Obara et al., 2004).

The hypotheses of this dissertation were that

- nerve injury might differentially influence the expression pattern of opioid receptors on primary sensory neurons in the DRGs, at the site of injury and at the peripheral nerve terminals in the paw skin

- the activation of opioid receptors expressed on primary sensory neurons, specifically nociceptors, results in an inhibition or reduction in the neuronal response to mechanical stimulation.

The specific goals were to investigate:

- whether mu-and delta-receptors are expressed in nociceptive primary sensory neurons following nerve injury;

- if the ligation influences the quantitative expression levels of mu-and delta-receptors in the DRG, at the ligation site and in the paw tissue;

- whether immune cells that accumulate around the ligation site contain opioid receptor agonists and CFR receptors;

- if and how the threshold, discharge rate and latency of response to mechanical stimulation of primary sensory neurons changes following nerve injury;

- if and how the application of DAMGO onto the receptive fields of primary sensory neurons from injured and uninjured nerves affects the threshold, discharge rate and latency of response to mechanical stimulation.

3 Animals, Materials and Methods

3.1. Animals

Experiments were performed in male C57BL/6J mice (25-30 g; 6-8 weeks old) bred at the Charité, Campus Benjamin Franklin, Forschungseinrichtung für experimentelle Medizin, Berlin. Animals were housed in groups of 6 per cage lined with ground corncob bedding. Standard laboratory rodent chow and tap water were available *ad libitum*. Room temperature was maintained at 22°C and the relative humidity was between 40% and 60%. A 12/12 hr (8 a.m. /8 p.m.) light/dark cycle was used. All experiments were performed according to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the local animal care committee (Landesamt für Gesundheit und Soziales (LAGeSo) Berlin, Germany).

3.2. Materials

3.2.1. Chemicals and reagents

Paraformaldehyde (PFA)	Sigma-Aldrich, St. Louis, MO
Gelatine	Sigma-Aldrich, St. Louis, MO
Chromium potassium sulphate	Sigma-Aldrich, St. Louis, MO
Hydrogen peroxide, H_2O_2 (30%)	Sigma-Aldrich, St. Louis, MO
Albumin solution	Sigma-Aldrich, St. Louis, MO
Glucose	Sigma-Aldrich, St. Louis, MO
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich, St. Louis, MO
Magnesium sulphate	Sigma-Aldrich, St. Louis, MO
Sodium phosphate	Sigma-Aldrich, St. Louis, MO
Sodium gluconate	Sigma-Aldrich, St. Louis, USA
CTOP (Cys2,Tyr3,Orn5,Pen7-amide)	Sigma-Aldrich, St. Louis, MO
DAMGO (D-Ala[2], N-MePhe[4], Gly-ol]-enkephalin	Bachem, Bubendorf, Switzerland
Saccharose	Carl Roth, Karlsruhe, Germany

Sodium chloride	Carl Roth, Karlsruhe, Germany
Entellan	Merck, Darmstadt, Germany
Mowiol	Merck, Darmstadt, Germany
2-methylbutane	Merck, Darmstadt, Germany
Phosphate buffered saline (PBS) tablets	Gibco, Invitrogen, Karlsruhe, Germany
Frozen section medium	Thermo Fisher Scientific Inc., Waltham, MA
Ethanol	J.T. Baker, Phillipsburg, NJ
Methanol	J.T. Baker, Phillipsburg, NJ
Xylene	J.T. Baker, Phillipsburg, NJ
Elite ABC kit (rabbit IgG)	Vector Laboratories, Burlingame, CA
DAB (3,3'-Diaminobenzidine) peroxidase substrate kit	Vector Laboratories, Burlingame, CA
Normal goat serum	Vector Laboratories, Burlingame, CA
Normal horse serum	Vector Laboratories, Burlingame, CA
Normal donkey serum	Jackson ImmunoReserach, Suffolk, UK
Forene (Isoflurane)	Abbott, Wiesbaden, Germany

3.2.2. Antibodies (Abs)

Primary Abs (all polyclonal)

Rabbit anti-mu opioid receptor	Abcam, Cambridge, UK
Rabbit anti-delta-opioid receptor	Gramsch, Schwabhausen, Germany
Guinea pig anti- α-CGRP	Bachem, Bubendorf, Switzerland
Chicken anti-NF200	Chemicon (now Millipore), Billerica, MA
Isolectin B4, Fluorescein-coupled	Sigma-Aldrich, St. Louis, MO
Rabbit anti-β-endorphin	Bachem, Bubendorf, Switzerland

Rabbit anti-Met-enkephalin	Bachem, Bubendorf, Switzerland
Goat anti–CRFR I/II	Santa Cruz Biotechnology, Santa Cruz, CA,

Secondary Abs

Texas Red-conjugated goat anti-rabbit	Vector Laboratories, Burlingame, CA
Texas Red-conjugated goat anti-guinea pig	Vector Laboratories, Burlingame, CA
Fluorescein-conjugated goat anti-guinea pig	Vector Laboratories, Burlingame, CA
Fluorescein-conjugated goat anti-chicken	Gene Tex Inc., Irvine, CA
Texas Red-conjugated donkey anti-rabbit	Jackson ImmunoReserach, Suffolk, UK
Fluorescein-conjugated donkey anti-goat	Jackson ImmunoReserach, Suffolk, UK

3.2.3. Technical devices

Anaesthesia machine	Datex-Ohmeda GmbH, Duisburg, Germany
Stereo microscope	Carl Zeiss AG, Oberkochen, Germany
Cryostat (Microm HM560)	MICROM International GmbH ,Walldorf, Germany
Fluorescence microscope (Axioskop2)	Carl Zeiss AG, Oberkochen, Germany
Lamp (ebq 100)	Leistungselektronik Jena GmbH, Jena, Germany

Devices for electrophysiological recordings

NeuroLog Case & Power Supply	Digitimer, Hertfordshire, UK
Headstage	Digitimer, Hertfordshire, UK
AC- Preamplifier (low noise, high input impedance, differential preamplifier)	Digitimer, Hertfordshire, UK
Audio Amplifier	Digitimer, Hertfordshire, UK
Filter	Digitimer, Hertfordshire, UK

Pulse buffer	Digitimer, Hertfordshire, UK
Pulse generator	Digitimer, Hertfordshire, UK
Stimulus Isolator	Digitimer, Hertfordshire, UK
PowerLab 4/26 (analog-digital converter)	ADInstruments, Oxfordshire,UK
TDS1002B oscilloscope	Tektronix, Berkshire, UK
Mehrkanaldosierpumpe, REGLO Digital MS-2/6	Ismatec, Glattbrugg, Switzerland
Discussion stereomicroscope	Leica, Wetzlar, Germany
Lamp KL2500	Leica, Wetzlar, Germany
Nanomotor	Kleindiek, Reutlingen, Germany

3.2.4. Other supplies

Microscope slides	R.Langenbrinck, Emmendingen, Germany
Cover slips	Menzel-Gläser, Braunschweig, Germany
Pipettes	Hirschmann, Eberstadt, Germany
Vortex mixer	Neolab, Heidelberg, Germany
Falcon tubes (5-25 ml)	Becton Dickinson, Franklin Lakes, NJ
Luer-Lok syringe	Becton Dickinson, Franklin Lakes, NJ
Needles	Becton Dickinson, Franklin Lakes, NJ
Surgical blades	Feather, Osaka, Japan
Biofuge fresco	Kendro, Langenselbold, Germany
Plastic tubes (0.5-2 ml)	Sarstedt, Nümbrecht, Germany
Nylon sutures (4-0 and 8-0)	Johnson & Johnson, Norderstedt, Germany
Von Frey filaments	Stoelting, Wood Dale, USA

3.2.5. Solutions

Phosphate buffered saline (1x PBS):   137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 2mM, KH_2PO_4, added up to 1L with distilled H_2O, pH 7.4

PBS+   1x PBS, 0.3% Triton X-100, 1% BSA

Fixation buffer: 4% PFA in 1xPBS, pH 7.4

Sucrose solution: 30 % sucrose in 1x PBS, pH 7.4

Gelatine solution: 1% gelatine and 0.1 % chromium potassium sulphate in distilled H_2O

Synthetic interstitial fluid (SIF) buffer: NaCl 123 mM, KCL 3.5 mM, $MgSO_4$ 0.7 mM, NaH_2PO_4 1.5 mM, $CaCl_2$ 2 mM Sodium gluconate 9.5 mM, Glucose 5.5mM Sucrose 7.5 mM, HEPES 10 mM, pH 8.2-8.4 with NaOH

3.3. Methods

For purchasing details on all reagents and Abs see paragraph 2.2.

3.3.1. Surgeries

All surgeries were performed under deep inhalational anesthesia. Animals were placed in a glass chamber on a ceramic perforated plate, which was located above tissues soaked with approximately 15 ml of isoflurane, until anesthesia was initiated. For the short-lasting (5 min) CCI of the sciatic nerve, the animals received a mouth piece that was filled with isoflurane-soaked cotton. For the longer-lasting CCI of the saphenous nerve (approximately 20 minutes), the animal was attached to an anesthesia machine and kept under the gaseous mixture of isoflurane (3-4%) and oxygen throughout the procedure. All experiments were performed at 2 days and 2 weeks or only at 2 weeks following surgeries.

CCI of the sciatic nerve

The CCI of the sciatic nerve, which innervates the hind paw, is a common and excessively studied model of neuropathic pain (Bennett, 1993; Bennett and Xie, 1988) and was used to evaluate the expression pattern of mu- and delta-opioid receptors in the DRGs, at the nerve injury site and within the hind paw tissue. The skin was cut at the level of the right mid-thigh, the underlying muscle was opened and the sciatic nerve exposed. Three loose silk ligatures (4/0) were placed around the nerve with about 1 mm spacing. They were tied until they elicited a brief twitch in the respective hind limb and the wound was closed with silk sutures, as described previously (Labuz et al., 2009).

CCI of the saphenous nerve

The saphenous nerve is commonly used in the in vitro skin-nerve preparation (Koltzenburg et al., 1997; Reeh, 1986). Compared to the sciatic nerve, the saphenous nerve is more convenient for such recordings because it is an exclusively sensory nerve without motor nerve impairment (Walczak et al., 2006). Since the saphenous nerve is much smaller than the sciatic nerve, the operation was conducted under a stereomicroscope (x25). A small incision was made on the anterior surface of the right thigh and the saphenous nerve was carefully freed from the connective tissue. Due to the small size of the nerve, no silk sutures but three very fine nylon sutures (8-0) were ligated loosely around the nerve with about 1 mm spacing and the wound was closed with two nylon sutures (4-0), according to Walczak et.al. (2006).

Sham surgeries

As controls, sham operations were performed on sciatic and saphenous nerves, respectively. The nerves were isolated by opening the skin and surrounding muscles according to A and B without performing ligations and the wound was closed (Labuz et al., 2009; Walczak et al., 2006).

3.3.2. Evaluation of mechanical hypersensitivity in vivo

The mechanical hypersensitivity was assessed in mice with a CCI or sham-surgery of the saphenous nerve and in naïve animals. The establishment of mechanical allodynia and hyperalgesia after a CCI of the sciatic nerve has been extensively studied and verified in our and other laboratories (e.g. Kabli and Cahill, 2007; Labuz et al., 2009; Obara et al., 2009; Truong et al., 2003). The testing was performed in plastic cages with a wire mesh bottom. Animals were habituated by placing them in the test cages three times for 15 min each day, starting one week before CCI throughout the duration of the experiments (2 weeks). Measurements were performed a day before (or immediately before) CCI and then 2 weeks later, using calibrated von Frey filaments. The plantar surface of the animal's hind paw was touched with the filament until it slightly bent, according to an up-down method (Labuz et al., 2009; Sommer and Schafers, 1998). Testing began with the 0.4 g (3.9 mN) hair. If the mouse responded to the stimulation of the footpad with a brisk withdrawal of the respective hind paw, the response was considered positive. In this case the next weaker hair was used; in case of a negative response, the next stronger hair was applied. The maximal number of applications was 6-9 and the cut-off was 4 g (39.2 mN). This procedure was performed according to our previous study (Labuz at al., 2009). A 50% threshold, which indicates the force of a von Frey hair at which an animal reacts in 50% of the presentations, was calculated using the formula:

50% threshold (g) = $(10^{[X_f + \kappa\delta]})/10{,}000$

indicating the force at which the individual mouse would withdraw the hind paw in 50% of trials (X_f=value (log units) of the final von Frey hair used, κ=tabular value according to Dixon, 1965; ∂=mean difference (log units) between stimuli), as described previously (Chaplan et al., 1994; Sommer and Schafers, 1998).

3.3.3. Tissue preparation

For immunohistochemistry and immunofluorescence the lumbar 4 and 5 DRGs and a part of the sciatic nerves (8-10 mm-long) from the ipsi- and contralateral sides to the CCI as well as subcutaneous tissue from both hind paws were isolated 2 and 14 days following CCI or sham operation. The part of the injured nerves included the ligation site and sites proximal and distal to it. Corresponding tissues were also obtained from naïve mice. Animals were deeply anesthetized with isoflurane and perfused transcardially with approximately 30 ml 1x PBS (pH 7.4) followed by 50 ml of ice-cold 1x PBS containing 4% PFA (pH 7.4). Paw tissue was dissected with a sharp razor blade and the sciatic nerves were removed after cutting the skin and muscles at the mid-thighs. Thereafter, the spinal column was cut and DRGs were harvested after removing the spinal cord. All tissues were postfixed for 2 h in 4% PFA in 1x PBS at 4°C, followed by placing them in a 30% sucrose solution (in 1x PBS) at 4°C overnight. On the next day, the tissues were frozen in a water-soluble frozen section medium. The DRGs were additionally (approximately 30 seconds) incubated in ice-cold 2-methyl-butane before the embedding. The embedded tissues were cut on a cryostat or kept at -80°C until further processing. 10 μm-thick sections were prepared from the DRGs and longitudinally cut sciatic nerves, while the paw tissue was cut into 12 μm-thick sections. The sections were mounted on gelatine-coated slides. These procedures were performed in accordance to Labuz et al. (2009).

3.3.4. Immunohistochemistry

Immunohistochemical staining of the sections was performed by incubating them for 45 min in 1x PBS with 0.5% H_2O_2 and 45% methanol to block endogenous peroxidase. To prevent nonspecific binding the sections were incubated for 60 min in 1x PBS containing 0.3% Triton X-100, 1% BSA and 5% goat serum. The sections were then incubated overnight with the polyclonal rabbit anti-mu-opioid receptor Ab (1:1500 in PBS+). On the next day, the slides were extensively washed (3-4 times for at least 10 min) with 1x PBS and further staining was performed with a vectastain avidin-biotin peroxidase complex according to the manufacturer's instructions using a goat anti-rabbit biotinylated secondary Ab and a avidin-biotin peroxidase (VECTASTAIN Elite) Kit. Finally, the sections were washed and stained with 3', 3'-diaminobenzidine tetrahydrochloride (DAB Substrate Kit) for 30 sec to 1 min. After the enzyme reaction the sections were washed in tap water, dehydrated in alcohol of increasing concentrations (70%, 80% and 100%), cleared in xylene solutions of increasing

concentrations (70%, 80% and 100%) and mounted in Entellan. This was performed according to a previous study (Mousa et al., 2001).

3.3.5. Single and double immunofluorescence

Sections were exposed to the blocking solution (1x PBS containing 0.3% Triton X-100, 1% BSA and 5% goat serum) for 1 h. For paw sections, 5% normal horse serum was additionally used. To examine single staining or the co-expression of mu- or delta-opioid receptors with neuronal markers, the sections were incubated overnight with polyclonal rabbit anti-mu- or anti-delta-opioid receptor Abs (1:800) alone or in combination with polyclonal guinea pig anti-α-CGRP (1:800) or chicken anti-NF200 (1:500). The next day, the slides were washed (3-4 times for at least 10 min in each case) and then incubated for 1 h with the following secondary Abs: goat anti-rabbit conjugated to texas red alone or combined with goat anti-guinea pig conjugated to FITC or goat anti-chicken conjugated to FITC (both at a dilution of 1:200). To identify opioid receptors in non-peptidergic C fibers, IB4 conjugated to FITC (1:150) was used according to the secondary antibodies. To examine the co-expression of opioid peptides with CRF receptors in immune cells, sciatic nerve sections were incubated with polyclonal rabbit Abs against β-endorphin or Met-enkephalin (both at 1:500) and polyclonal goat anti–CRF receptors (CRFR) recognizing both CRFR1 and CRFR2 (1:400), followed by the secondary Abs donkey anti-rabbit conjugated to Texas red and donkey anti-goat conjugated to FITC (1:200), similar to opioid receptor staining. Thereafter, the sections were washed with PBS, mounted in Mowiol and viewed under a fluorescence microscope (Zeiss) with appropriate filters.

Specificity of the staining by opioid receptor Abs included omission of the primary Abs and preabsorption of primary Abs with the respective immunizing peptides. These were added to the above-mentioned primary Ab solution in a 5-fold and 10-fold excess and left on a shaker for at least 3 h before applying the solution to the tissue. The control experiments did not show opioid receptor or opioid peptide and CRFR staining. The procedures were performed according to our previous study (Labuz et al., 2009).

3.3.6. Electrophysiology

Electrophysiological recordings were carried out to examine the thresholds, the discharge rates and the latency of sensory primary afferents in response to different strengths of mechanical stimulations. Furthermore, the effects of the mu-receptor agonist DAMGO on

these parameters were investigated. Consistently with the immunohistochemical experiments we attempted to perform electrophysiological recordings at 2 days and 2 weeks following CCI of the saphenous nerve. However, we were unable to record action potentials from sensory fibers at 2 days after CCI, in line with the report by Walczak et al. (2006) and analyzed in the discussion (5.2). Therefore, the following experiments were performed at 2 weeks after CCI or sham surgeries as well as in non-operated nerves. Mice were killed with an overdose of isoflurane and the hair on the respective hind legs and paws was removed. A small needle was used to fix the hind paw onto a silicon rubber bottom of a Petri dish. An incision was made at approximately 1–2 mm above the knee joint and the skin was opened along the entire saphenous nerve up to the inguinal ligament. The exposed nerve and muscle were frequently rinsed with oxygenated SIF throughout the duration of the preparation. To obtain the paw skin with the attached nerve, the most distal toe phalanges were cut and the skin on the plantar side of the paw and leg was cut open towards the inguinal region. Then, starting at the remaining phalanges and using very fine scissors, the hairy skin was separated from the subcutaneous tissue and pulled slowly backwards until the point where the saphenous nerve enters the skin. The remaining part of the nerve was freed from the surrounding muscles and blood vessels and the obtained skin-nerve preparation was placed "inside-up" in an organ bath to facilitate oxygenation through the corium side of the skin. The bath was constantly perfused with oxygenated and warmed (at approximately 30°C) SIF buffer at a flow rate of 15 ml/min that maintained the skin-nerve preparation viable for up to 6 or 7 h. These procedures were performed according to previous studies using rat or mouse saphenous nerves (Koltzenburg et al., 1997; Reeh, 1986).

3.3.7. In vitro skin-nerve recordings

The skin was placed with the corium side up and pinned with insect needles to a transparent silicon rubber bottom in the organ bath. The attached saphenous nerve was pulled through a small opening into the second bath chamber and laid on a small mirror-based platform, which served as a dissection plate (Fig. 3.1). To provide electrical isolation the chamber was filled with mineral oil. The nerve trunk was desheated by pulling off (over 4 to 6 mm) the perineurium. Small filaments were teased from the desheathed nerve and attached to the recording electrode. The filaments were divided until only one or a few single sensory neurons could be recorded (care was taken that the filaments had no overlapping receptive fields). Individual units could usually be distinguished from each other based on difference in the amplitudes and shapes of the action potentials. The receptive fields of primary afferent

fibers were identified by manually probing the skin with a glass rod that is known to activate around 80% of cutaneous afferent fibers in a rat skin-saphenous nerve preparation (Kress et al., 1992). Only signals larger than twice the noise level were accepted for further analysis. The reference current (measured by a reference electrode in the SIF buffer) was subtracted from the signal. Action potentials from single sensory neurons were recorded extracellularly with a low-noise differential amplifier. The amplified signals were visualized on an oscilloscope demonstrating the shape and width of the action potential, and then on the computer with the help of an analog-digital converter (Fig. 3.1). Off-line analysis, using Chart v6 and spike Histogram extension software, enabled the identification of action potentials from single sensory fibers and their evaluation with respect to the discharge rate and latency to mechanical stimulation.

The conduction velocity of each axon was determined by electrically stimulating the receptive field with suprathreshold current pulses with durations of 50, 150, or 500 μs using a sharp tungsten metal electrode. The conduction velocity was calculated as the distance from the receptive field to the recording electrode (in millimeters) divided by the electrical latency of the action potential (Koltzenburg et al., 1997; Milenkovic et al., 2008). The electrical latency is the time difference (in milliseconds) between the onset of the electrically-evoked stimulus and the onset of the responding action potential. Fibers that conducted faster than 10 m/s were classified as Aβ fibers, those conducting 1.2 – 10 m/s as Aδ, and those conducting slower than 1.2 m/s as C fibers, as proposed earlier for mice (Koltzenburg et al., 1997). Next, all sensory fibers were characterized by assessing their mechanical thresholds by von Frey hair stimulation of the most sensitive spot of the receptive field. If possible, the computer-controlled nanomotor was subsequently manoeuvred onto this spot and a computer-written protocol was run to obtain discharge patterns of Aβ, Aδ and C fibers that allowed the further classification of the sensory fiber subtypes. Thus, Aβ fibers were classified either as rapidly adapting mechanoreceptors (RAMs) or as slowly adapting mechanoreceptors (SAMs), while Aδ fibers were classified as D-hairs or as Aδ fiber mechanonociceptors (AMs) (Koltzenburg et al., 1997; Lewin and Moshourab, 2004). Moreover, discharge rates and the latency to mechanical stimulation of different intensities could be assessed (see below).
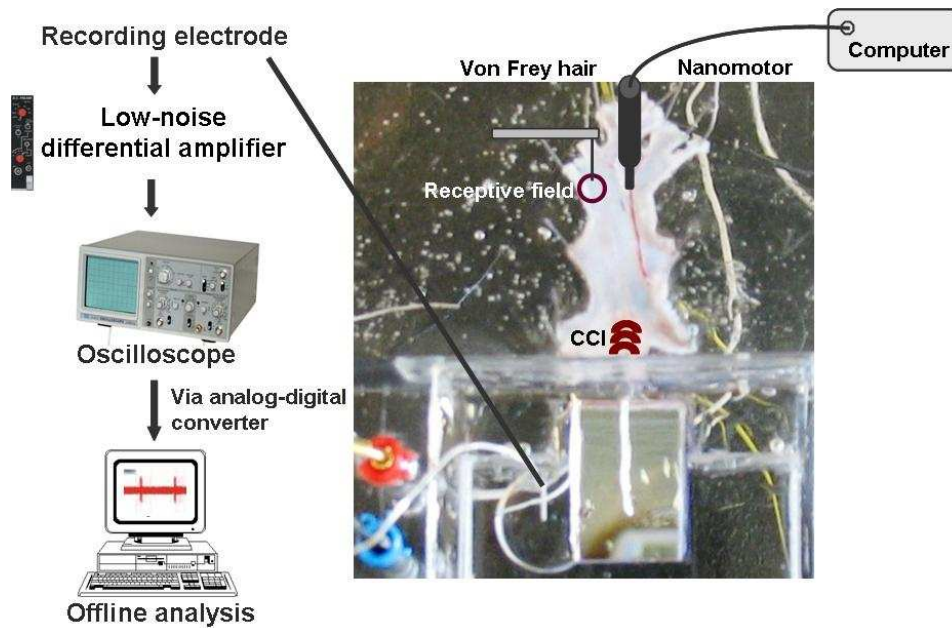
Fig. 3.1: Scheme of the skin-nerve preparation and electrophysiological recordings. Mechanical stimulation of a single fiber was done using von Frey hairs of different strength to determine the fiber's threshold as well as using a computer-controlled nanomotor to evaluate the discharge pattern, the rate and the latency to the first action potential. An action potential of a fiber was recorded and amplified with a low-noise differential amplifier, filtered and identified on the oscilloscope and on a computer equipped with an appropriate program for offline analysis.

3.3.7.1 Determination of sensory fibers' mechanical thresholds

The mechanical thresholds of single sensory fibers were determined using calibrated von Frey hairs, as reported in many previous in vivo studies and in in vitro skin-nerve preparations (Koltzenburg et al., 1999; Koltzenburg et al., 1997; Kumazawa and Perl, 1977; Lynn and Carpenter, 1982). The von Frey filaments were the same as those used in the behavioural in vivo studies. The range of von Frey filaments used was 0.007 g – 4.55 g. Testing began with a 0.13 g hair and if the fiber responded with an action potential a lower hair was applied. If the fiber did not respond the next stronger hair was used. The force of the lowest fiber, which upon a slight bending evoked an action potential, was defined as the minimal force necessary to mechanically stimulate the fiber, as defined by others (Koltzenburg et al., 1997). The mechanical threshold was expressed in grams.

3.3.7.2 Determination of sensory fibers' discharge pattern and rates

To determine the discharge pattern and rate as well as the latency of sensory fibers to mechanical stimulation of different intensities, a nanomotor was used, as reported previously (Milenkovic et al., 2008). The nanomotor is a metal probe attached to a lineary stepping motor under computer control. The metal probe consists of a stator attached to a slider by

friction. The stator expands and contracts to applied voltage. The voltage pulses change the friction between the slider and the stator that allows for a stable and accurate up- or down-movements of the slider. The nanomotor was used to deliver standardized increasing displacement stimuli at regular intervals to the fibers' receptive fields. After determining the mechanical threshold with von Frey hairs, the nanomotor was manoeuvred onto the fiber's receptive field. The movements of the nanomotor were applied in steps, with the lowest number of 10 steps, which corresponds to approximately 12 μm. Using small movements (usually 96 μm) of the nanomotor, the mechanical stimulus was advanced onto the receptive field until one action potential was evoked. The amplitude of the stimulus was then systematically reduced and the probe moved into a position, at which the smallest stimulus possible (12 μm) reliably evoked at least one action potential when the probe was advanced. The starting position of the mechanical stimulator was therefore just above the threshold for each recorded unit. The sensory nerve fiber was then confronted with an ascending series of displacement stimuli, sent as a pre-programmed series of commands to the nanomotor (Milenkovic et al., 2008). For the first standardized displacement stimuli, the nanomotor went 10 steps downwards for 10 seconds and 10 steps up again, followed by an interstimulus period of 30 seconds. Then, a second displacement was applied, which consisted of a downward movement of 24 μm (20 steps), increasing the step size in a logarithmic manner to the last (seventh) displacement of 768 μm (640 steps). Each displacement stimulation lasted 10 seconds and was followed by the 30 second interstimulus period. A stimulation length of 10 seconds was chosen, because electrical recordings in humans and rats indicated that many C fibers respond to noxious pressure with a dynamic discharge lasting about 10 seconds, followed by slowly adapting tonic discharges, whereof the dynamic responses encoded the stimulus strength (Adriaensen et al., 1984; Handwerker et al., 1987b). Based on the discharge pattern to all displacement stimuli, Aβ fibers were sub-classified into RA and SA fibers, while Aδ fibers were subclassified into D-hair and AM nociceptors. The discharge rate (number of action potentials per 10 seconds of stimulation) was calculated offline with LabChart v6 and spike Histogram extension software.

3.3.7.3 Determination of the sensory fibers' latency to response

The mechanical latency (in milliseconds) was calculated offline. It was measured for each individual recorded afferent by measuring the delay between the onset of each displacement stimulus and the first spike of the fiber minus the conduction delay measured for the same fiber (Milenkovic et al., 2008).

3.3.7.4 Assessment of DAMGO effects

Two protocols were used to assess the effects of DAMGO on mechanically evoked sensory fiber responses. The first protocol focused on the mechanical thresholds and was conducted as follows: After determination of the fiber's baseline mechanical threshold with von Frey hairs, the receptive field was sealed with a metal ring (approximately 6 mm in diameter) and the SIF buffer inside a ring was replaced for 2 minutes with 100 µl of prewarmed (about 30°C) SIF buffer containing DAMGO (100 µM/100 µl) or only SIF buffer (100 µl) as a control. Afterwards, the ring was emptied of the DAMGO or of the buffer solution, and the fiber's mechanical threshold was again tested with von Frey hairs. Next, the fiber's receptive field was washed for 10-15 minutes and the mechanical threshold was assessed the third time. If the application of DAMGO or of the buffer had resulted in a change of the mechanical threshold, the receptive field was sealed again with the metal ring after the washout period and filled with 100 µl of prewarmed (about 30°C) SIF buffer containing an equimolar concentration of CTOP (100 µM), a selective mu-opioid receptor antagonist, for 3 minutes, followed by a 2 minutes incubation with 100 µl of prewarmed SIF buffer containing DAMGO (100 µM). Afterwards, the mechanical threshold was tested the fourth time. The application of CTOP was used to examine the mu-opioid receptor selectivity of the effect of DAMGO. The dose of 100 µM of DAMGO was the most effective in our pilot experiments testing it in a dose range of 1-500 µM.

The second protocol was accomplished as follows: After determining the fiber's mechanical threshold with von Frey hairs, the nanomotor was positioned above the fiber's receptive field and the computer-controlled protocol was run (see 3.3.7.2). Then, DAMGO (100 µM/100 µl) or buffer (100 µl) were applied to the receptive field (as described above) for 2 minutes and removed afterwards. The mechanical threshold was tested again and subsequently, the nanomotor protocol was run the second time. It was only very rarely possible to run the nanomotor a third time after a washout period of 10-15 minutes, as not all fibers responded to the mechanical stimulation until the end of the testing (presumably they did not survive until the end of the procedure due to the longer-lasting stimulation). For the same reason, it was not possible to test the nanomotor protocol on all sensory afferent fibers identified. Therefore, sometimes only von Frey hair stimulation was used, especially in the end of an experiment, when the number of fibers that were still alive began to decrease.

3.4. Data acquisition

3.4.1. Quantification of opioid receptor expression

DRG

Quantification of opioid receptor-expressing DRG cells was performed in accordance to the previous study by Mousa et al. (2007). The lumbar 4 and 5 DRGs from five to six animals were used for each condition (i.e. naïve, sham-operation (ipsi- and contralateral) and CCI (ipsi- and contralateral)). For each animal, every second section of each DRG that was serially cut at 10 μm was stained for mu- or delta-opioid receptors, respectively, resulting in three stained slides (each containing approximately 6 slices) for each receptor. For neuronal counting, pictures were taken from two clearly stained DRG slices from each slide using the AxioVision program (Zeiss) and a microscope (Zeiss Axioskop 2) with 20x objectives using light (mu-receptor) or fluorescence with appropriate filters (delta-receptor). The density threshold for positive immunoreactive DRG cells was determined by averaging the intensity staining from 5-6 cells that were judged to be minimally positive, in each section. Using the cell counter application of the graphic program ImageJ, the total number of mu- or delta-immunoreactive neurons per each picture was counted by the examiner unaware of the condition. Thus, for each condition (i.e. naïve, sham-operation (ipsi- and contralateral) and CCI (ipsi- and contralateral)), 6 slices per animal from five to six mice were analyzed. First, the number of positively-stained cells was averaged for each animal and these values were used for the statistical evaluation. The data are expressed as the percentage of total counted DRG neurons according to the formula: % = (positive cells/total counted cells) x 100.

Sciatic nerves

Expression of mu- and delta-receptors in the sciatic nerve was assessed by measuring the intensity of the staining (i.e. by counting the number of positively-stained pixels) and, when possible, by counting the number of positively-stained fibers. Every third section of the sciatic nerve that was serially cut at 10 μm for each animal (n = 5-6) was stained for mu- or delta-receptors, respectively, resulting in two stained slides (each containing 4-6 slices) for each receptor. Two pictures per slide were taken from each investigated site of CCI sciatic nerves (directly distal and proximal to the ligation, as well as the adjacent further proximal site; each about 700 μm long) using the AxioVision program (Zeiss) and the 20x objectives of a fluorescence microscope (Zeiss Axioskop 2) with appropriate filters. Similarly, two pictures

per slide were taken from the corresponding regions of the contralateral nerves of CCI animals, both nerves of sham-operated and nerves of naïve animals.

Quantification was done using the graphic program ImageJ. The pictures were opened in the RGB (additive colour model) mode, but as the sciatic nerves were stained with Texas Red, only the red channel was used. The background staining was measured with ImageJ and subtracted from the opioid receptor staining. The threshold density ranges were adjusted to encompass and match the immunoreactivity (red fluorescence), so that all immunoreactivity within the threshold appeared in white pixels, and non-immunoreactive (below the threshold) material as black pixels. The same threshold was set for all pictures. A standardized box was positioned over the stained part of the pictures (marking a constant area of approximately 0.2 mm^2) and the number of positively-stained (white) pixels within the area was calculated by the program, as previously (Mousa et al., 2007). In parallel, the number of opioid receptor-stained fibers was quantified. For each condition (i.e. naïve, sham-operation (ipsi- and contralateral) and CCI (contra- and ipsilateral: directly and further proximal and distal)), 4 slices per animal from five to six mice were analyzed. First, the number of positively-stained pixels or fibers was averaged for each animal and these values were used for the statistical evaluation. The examiner was unaware of the identity of the pictures from the nerves of naïve and sham-operated mice as well as from nerves contralateral to the CCI. Because the ligature in CCI-operated nerves was visible, it was not possible to blind the CCI nerves. However, to minimize possible bias, the CCI nerves stained for mu- or delta-receptors were blinded and quantified in parallel.

Paw tissue

Mu-and delta- receptor immunoreactive fibers were quantified in accordance to a previous study (Mousa et al., 2002). The paw tissue of five to six animals was used for each condition (naïve, sham-operation (ipsi-and contralateral) and CCI (ipsi-and contralateral)). For each animal, every second section of the paw tissue that was longitudinally and serially cut into 12 μm-thick sections was stained for mu- or delta-receptors, respectively, resulting in three stained slides (each containing 4-6 slices) for each receptor. For neuronal fiber counting, pictures were taken from two clearly stained paw tissue sections from each slide using the AxioVision program (Zeiss) and a fluorescent microscope (Zeiss Axioskop 2) with 20x objectives and appropriate filters. A rectangular box of constant size was placed over the immunostained area. The box size was based on an averaged area from about 4 mu- and delta-receptor-stained pictures showing immunoreactive fibers, and calculated 0.19 mm^2 for mu-

receptors and 0.22 mm^2 for delta-receptors. For each condition (i.e. naïve, sham-operation (ipsi- and contralateral) and CCI (ipsi- and contralatera), 6 slices per animal from five to six mice were analyzed. First, the number of positively-stained fibers was averaged for each animal and these values were used for the statistical evaluation. The observer was blinded to the condition during the data acquisition.

3.4.2. Electrophysiology

The von Frey mechanical thresholds of all fibers at all experimental conditions (baseline, after DAMGO application, after washout and after CTOP and DAMGO) were defined in grams of the lowest von Frey hair that evoked an action potential when slightly bent on the fiber's receptive field. The mean mechanical threshold was calculated for all fibers of each fiber type (Aβ, Aδ and C fibers) per condition (the respective fiber number is presented in the results). All data from the nanomotor testing were collected with the use of a data acquisition software (LabChart v6, including spike Histogram extension software) run on a PC, and action potentials were subsequently analyzed. For evaluation of the discharge rates to mechanical stimulation, all action potentials were counted at a time period of 10 seconds starting with the onset of a displacement stimulus. This time window contained all the spikes during the rise time, plateau and release of the stimulus, as described earlier (Koltzenburg et al., 1997). The mean discharge rate was calculated per each displacement and per each condition for all fibers of each fiber type (SAM, RAM, D hairs, AMs and C fibers) (the respective fiber number is stated in the results). The latency to the fiber's first action potential following mechanical stimulation was calculated in milliseconds, in a similar manner as described for the discharge rate.

3.5.   Statistical data analysis

All data were analyzed using SigmaPlot 10.0, including SigmaStat and are expressed as means and the standard error of the means (mean ± SEM). Expression of opioid receptors was examined by a one-way repeated measurement (RM) analysis of variance (ANOVA) followed by the Bonferroni test (normally distributed data), or by a one-way ANOVA on ranks (not normally distributed data). In in vivo experiments evaluating mechanical hypersensitivity, data were assessed with a two-way RM ANOVA followed by the Bonferroni test. In electrophysiological experiments, two sample comparisons of respective fiber types from sham-operated versus non-operated or injured versus uninjured nerves, as well as of pre-

and post threshold values following buffer treatment were assessed by t-test (independent, normally distributed data), Mann-Whitney test (independent, not normally distributed data) or Wilcoxon Test (dependent, not normally distributed data). Multiple comparisons of threshold values after DAMGO and DAMGO/CTOP treatment were evaluated by a one-way RM ANOVA on ranks and Tukey test (not normally distributed data). All multiple comparisons of the nanomotor data (discharge rates and latencies) were analyzed with a two-way RM ANOVA followed by the Bonferroni test. The data were defined as statistically significant, when $p < 0.05$ and presented in the graphs as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4 Results

4.1. Co-expression of peripheral mu- and delta-opioid receptors with sensory nerve fiber markers following nerve injury

The expression pattern of peripheral mu- and delta-receptors was investigated in three different regions innervated by the injured or non-injured sciatic nerve. These regions include the DRG, where opioid receptors are synthesized, the sciatic nerve along which the receptors are transported, and the hind paw, where opioid receptors are delivered to and are present at the peripheral terminals of sensory neurons (Hassan et al., 1993; Stein et al., 1990). To identify the sensory neuron types expressing mu- and delta-receptors we employed the following neuronal markers: CGRP recognizing peptidergic C and A fibers, IB4 binding to nonpeptidergic C fibers, and NF200 that stains myelinated Aβ and Aδ fibers. Double-immunofluorescence was used to co-stain mu- or delta-receptors with each of the above mentioned neuronal markers in the DRG. In the sciatic nerves, opioid receptors were co-stained with IB4 and CGRP, but not with NF200. NF200 exhibited a strong uniform staining resulting from the uniformly distribution and large size of the myelinated Aβ and Aδ fibers, which made single fibers indistinguishable in the sciatic nerve. As the cell bodies and its axons showed a similar staining pattern, we did not additionally test the co-expression in the terminal endings of axons within the paw. The experiments were performed at 2 days and 2 weeks after CCI, representing early and advanced stages of neuropathic pain, respectively (Bennett and Xie, 1988; Labuz et al., 2009). However, due to a similar pattern of co-labelling at both time points in all conditions, the results are presented for 2 days following nerve injury only.

4.1.1. Co-expression in the DRG

Figure 4.1 shows numerous small- to medium-diameter DRG cells co-expressing mu- or delta-receptors with CGRP, suggesting that opioid receptors are located mainly in peptidergic C and Aδ neurons in the DRG.
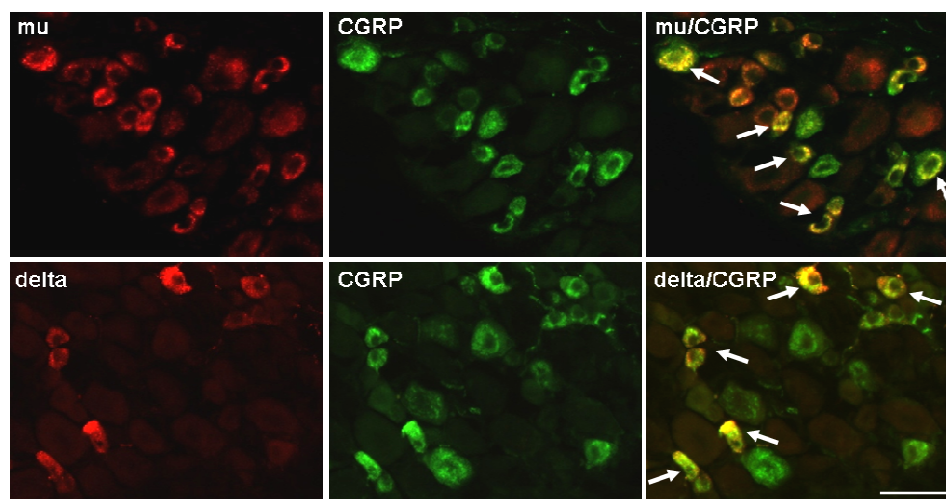
Figure. 4.1: Co-expression of mu- or delta-receptors and CGRP in DRGs. Representative double-immunofluorescence images showing co-expression of mu- (upper panel) or delta- (lower panel) receptors and CGRP in DRGs ipsilateral to the injured sciatic nerve, at 2 days following CCI. Arrows mark cells co-expressing opioid receptors and CGRP. The bar represents 50 µm.

Very few DRG cells expressing mu- or delta-receptors were co-stained with IB4 (Fig. 4.2), indicating a sparse expression of opioid receptors in non-peptidergic small diameter DRG neurons.
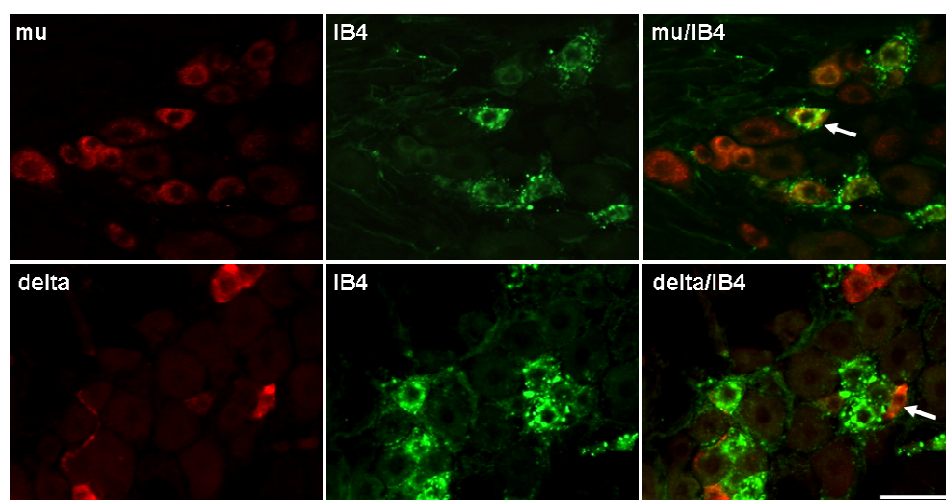
Figure 4.2: Co-expression of mu- or delta-receptors and IB4 in DRGs. Representative double-immunofluorescence images showing co-expression of mu- (upper panel) or delta- (lower panel) receptors and IB4 in DRGs ipsilateral to the injured sciatic nerve, at 2 days following CCI. Arrows mark cells co-expressing opioid receptors and IB4. The bar represents 50 µm.

Similarly, very few DRG cells co-expressing mu- or delta-receptors and NF200 were found (Fig. 4.3), pointing to a minor presence of opioid receptors in myelinated DRG neurons. Together, mu- and delta-receptors are located mainly in peptidergic C and Aδ neurons in the DRG at 2 days and 2 weeks (data not shown) after nerve injury.
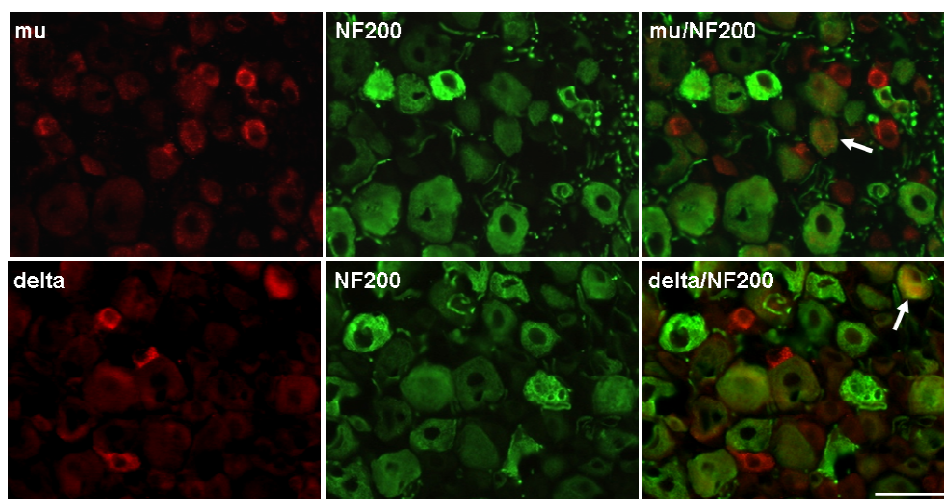
Figure 4.3: Co-expression of mu- or delta-receptors and NF200 in DRGs. Representative double-immunofluorescence images showing co-expression of mu- (upper panel) or delta- (lower panel) receptors and NF200 in DRGs ipsilateral to the injured sciatic nerve, at 2 days following CCI. Arrows mark cells co-expressing opioid receptors and NF200. The bar represents 50 μm.

4.1.2. Co-expression in the sciatic nerve

Double-immunofluorescence staining in sciatic nerves revealed that mu- and delta-receptors are strongly expressed in CGRP-positive, peptidergic fibers (Fig. 4.4) and to a much lower degree in IB4-binding, non-peptidergic fibers (Fig. 4.5). Taken together, these results suggest that mu- and delta-receptors are mainly expressed in peptidergic C and A fibers in the sciatic nerve at 2 days and 2 weeks (data not shown) after nerve injury, similar to the findings in the DRG.
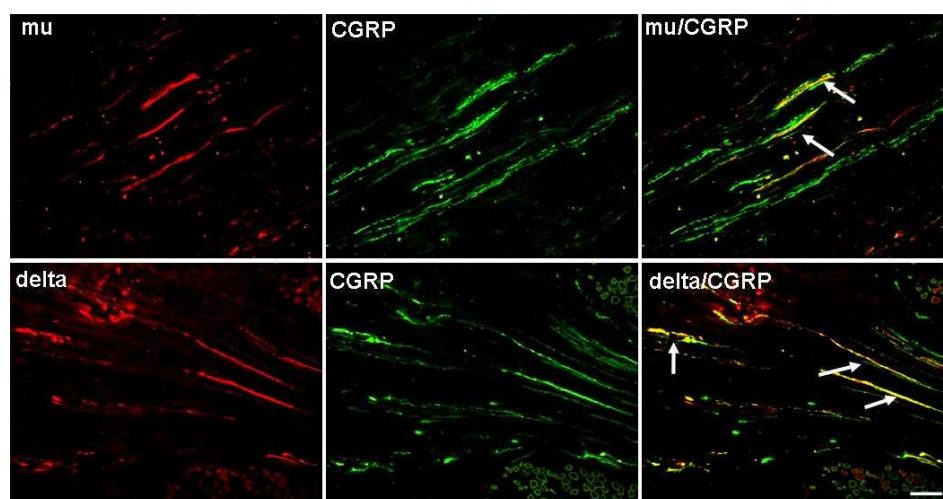
Figure 4.4: Co-expression of mu- or delta-receptors and CGRP in sciatic nerves. Representative double-immunofluorescence images showing co-expression of mu- (upper panel) or delta- (lower panel) receptors and CGRP proximally to the ligature in injured sciatic nerves, at 2 days after CCI. Arrows mark fibers co-expressing opioid receptors and CGRP. The bar represents 50 μm.
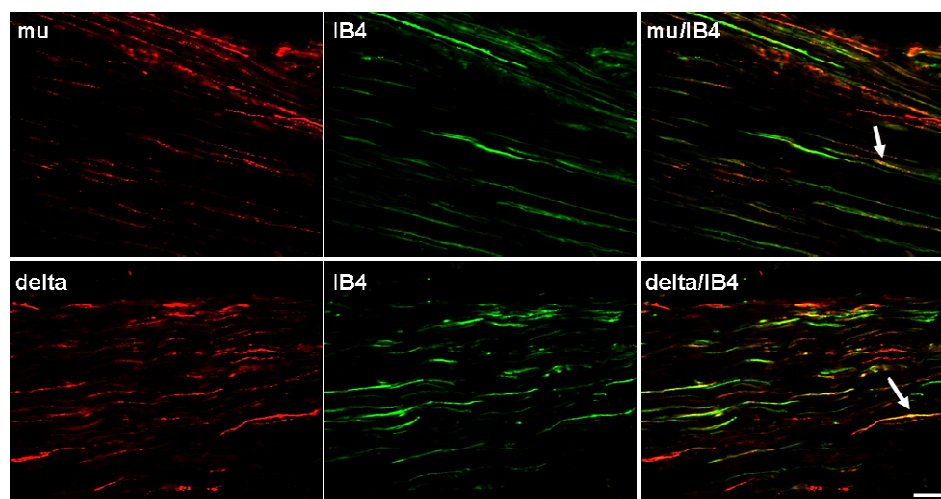
Figure 4.5: Co-expression of mu- or delta-receptors and IB4 in sciatic nerves. Representative double-immunofluorescence images showing co-expression of mu- (upper panel) or delta- (lower panel) receptors and IB4 proximally to the ligature in injured sciatic nerves, at 2 days after CCI. Arrows point to fibers co-expressing opioid receptors and IB4. The bar represents 50 μm.

4.2. Co-expression of β-endorphin and Met-enkephalin with CRF receptors in immune cells at the injured sciatic nerve

Natural ligands of opioid receptors are endogenous opioid peptides such as β-endorphin and Met-enkephalin. Immune cells accumulating at the injured sciatic nerves were identified as a source of both opioid peptides under neuropathic pain conditions (Fig. 4.6). Following the notion that CRF can release opioid peptides from immune cells to decrease neuropathic pain (Labuz et al., 2009), β-endorphin and Met-enkephalin were found to be co-expressed with CRF receptors in leukocytes accumulating at the injured nerve at 2 days (Fig. 4.6) and 2 weeks (data not shown) following CCI.

Figure 4.6 (next page): Co-expression of opioid peptides and CRF receptors at the injury site in sciatic nerves. Representative double-immunofluorescence images showing co-expression of β-endorphin (END; upper panel) or Met-enkephalin (ENK; lower panel) opioids and CRF receptors (CRFR) in immune cells accumulating around the injured sciatic nerves, at 2 days following CCI. Arrows point to cells co-expressing opioid peptides and CRFR. The bar represents 50 μm.
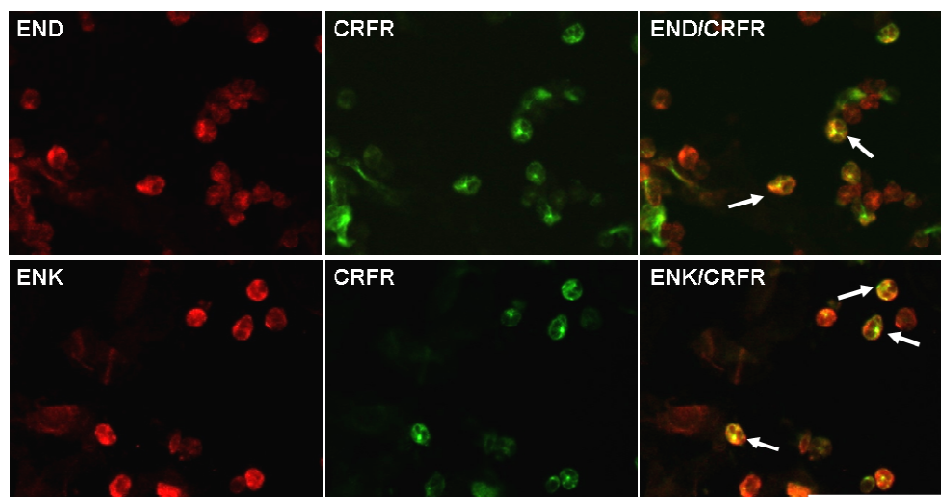
4.3. Quantification of peripheral mu- and delta-opioid receptor expression following nerve injury

Peripheral mu- and delta-receptors were quantified in the DRGs, the sciatic nerves and the hind paws at 2 days and 2 weeks following CCI. As controls, the corresponding tissues from naïve mice and those at 2 days and 2 weeks after sham-surgery were analyzed. In addition, corresponding tissues contralateral to the CCI or to sham-surgery were assessed.

4.3.1. Mu- and delta-receptor expression in the DRG

Mu-receptors were expressed predominately in small- to medium-size neurons in the DRGs of naïve mice, as well as in the DRGs ipsilateral to the CCI at 2 days and 2 weeks following nerve injury (Fig. 4.7 A). A similar expression pattern was found in DRGs contralaterally to the CCI as well as in DRGs of sham-operated mice, at both time points after surgeries (data not shown). Quantitative analysis revealed that 36.1 ± 1.6 % of all DRG cells expressed mu-receptors in naïve animals. Neither a sham-operation nor CCI resulted in a significant change in the number of mu-receptor expressing cells, at 2 days and 2 weeks following surgeries ($p > 0.05$, one-way RM ANOVA; Fig. 4.7 B).

Figure. 4.7: Expression of mu-receptors in DRGs following sciatic nerve injury.
(A) Representative immunohistochemical images showing mu-receptor immunoreactive neurons (dark brown) in DRGs of naïve mice and in DRGs ipsilaterally to the injury at 2 days and 2 weeks following CCI. The bar represents 50 µm.
(B) Quantitative analysis of mu-receptor expression in the DRGs of naïve mice, in the DRGs ipsilaterally (ipsi) and contralaterally (contra) to sham-surgery or to CCI, at 2 days and 2 weeks following surgeries. The data represent the percentage of mu-immunoreactive DRG cells, and are expressed as mean ± SEM. N = 5-6 mice per group. There were no statistically significant differences among treatments ($p > 0.05$, one-way RM ANOVA).

Delta-receptors were, similar to mu-receptors, predominately expressed in small- to medium-size neurons in DRGs of naïve mice, as well as in DRGs ipsilateral to the CCI at 2 days and 2 weeks following nerve injury (Fig. 4.8 A). The expression pattern in DRGs contralateral to the ligated nerves and in DRGs of sham-operated nerves was similar (data not shown). Delta-receptors were found to be expressed in 13,3 ± 0,9 % of all DRG cells in naïve animals and this number was similar in the ipsi- and contralateral DRGs of sham-operated and CCI animals. Overall, there were no statistically significant differences among the treatments ($p > 0.05$, one-way RM ANOVA; Fig. 4.8 B).

Figure. 4.8: Expression of delta-receptors in DRGs following sciatic nerve injury.
(A) Representative immunofluorescence images showing delta-receptor immunoreactive neurons (bright red) in DRGs of naïve mice and in DRGs ipsilaterally to the injury at 2 days and 2 weeks following CCI. The bar represents 50 µm.
(B) Quantitative analysis of delta-receptor expression in the DRGs of naïve mice, in the DRGs ipsilaterally (ipsi) and contralaterally (contra) to sham-surgery or to CCI, at 2 days and 2 weeks following surgeries. The data represent the percentage of delta-immunofluorescent DRG cells, and are expressed as mean ± SEM. N = 5-6 mice per group. No statistically significant differences were found (p > 0.05, one-way RM ANOVA).

4.3.2. Mu- and delta-receptor expression in the sciatic nerves

As presented in Fig. 4.9 A, a strong accumulation of mu-receptor immunoreactivity was found directly proximal to the most proximal ligature, both at 2 days and at 2 weeks following CCI. The mu-receptor expression was evaluated with regard to the intensity of the staining and, when possible, to the number of positively stained fibers in an area of approximately 0.2 mm^2. Quantitative analysis showed a robust and significantly higher intensity of mu-receptor staining directly proximally to the ligatures at 2 days and 2 weeks after CCI as compared to all other conditions (i.e. to areas located further proximally and distally to CCI or to nerves of naïve and sham-operated mice) (p < 0.001, one-way RM ANOVA and Bonferroni t-test; Fig. 3.9 B). At 2 days following CCI there was a slight increase in mu-receptor staining in the region further proximally to the ligatures that was

significantly different when compared to the distal region ($p < 0.05$, one-way RM ANOVA and Bonferroni t-test; Fig. 4.9 B). Otherwise, there was a comparable intensity of mu-receptor staining in nerves of naïve and sham-operated mice as well as in the contralateral nerves to the CCI and sham operation, at 2 days and 2 weeks after surgeries.

We also attempted to quantify the number of mu-receptor positive sciatic nerve fibers. This could not be done directly proximally to the nerve ligation, as the staining was too intense to differentiate between the fibers (Fig. 4.9 A). However, analysis of the other sites revealed that there were no significant differences in the number of mu-receptor-immunoreactive sciatic nerve fibers among naïve, sham-operated and CCI animals at 2 days ($p > 0.05$, one-way RM ANOVA) and 2 weeks ($p > 0.05$, one-way RM ANOVA on ranks) following surgeries (Fig. 4.9 C).
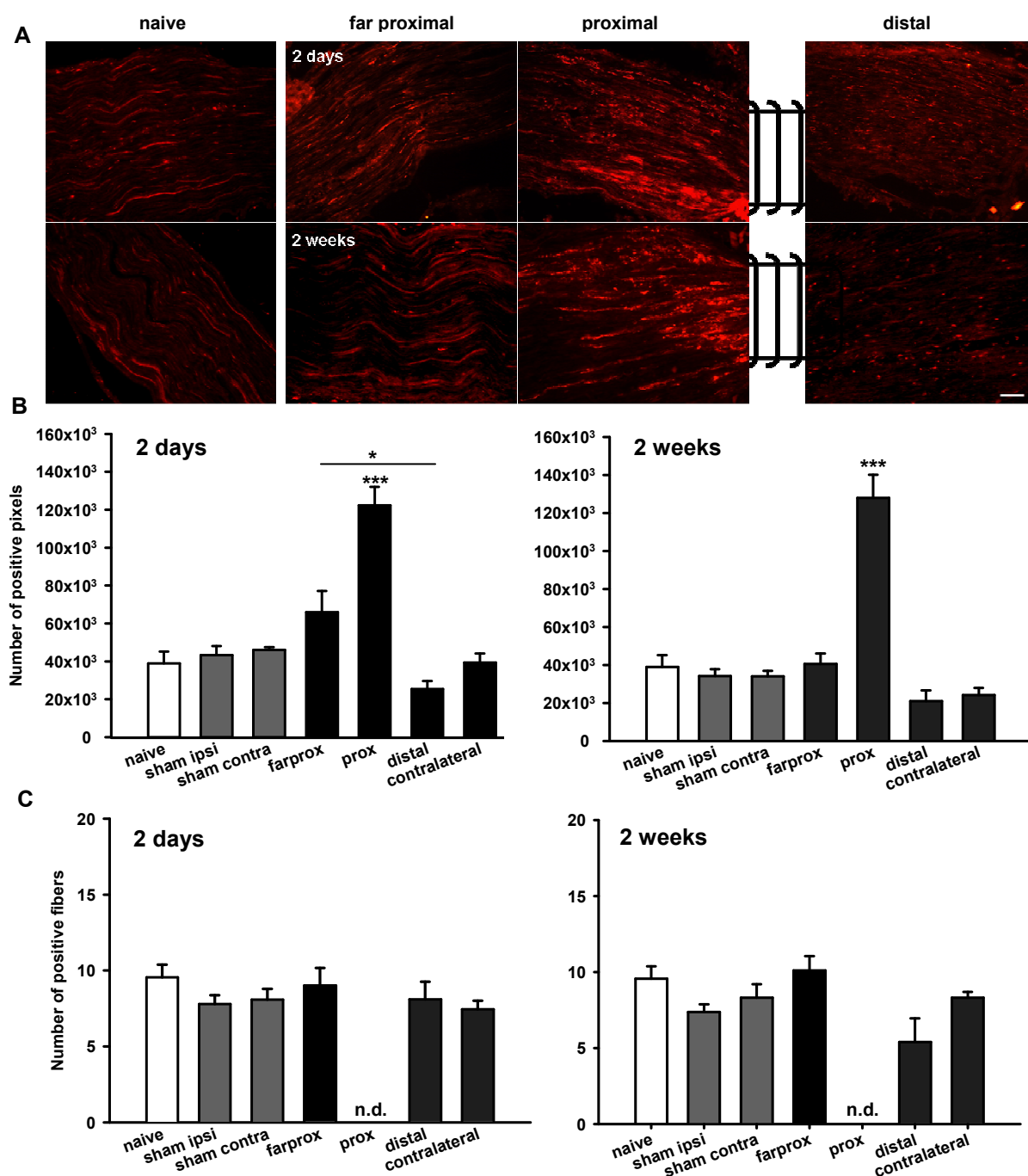
Figure. 4.9: Expression of mu-receptors in the sciatic nerves.
(A) Representative immunofluorescence images showing mu-receptor expression in the sciatic nerve of naïve mice and in the injured nerves at 2 days and 2 weeks following CCI. The images show areas immediately proximal (about 0-700 μm) and further proximal (about 700-1400 μm) to the most proximal ligature, as well as the area immediately distal to the most distal ligature (about 0-700 μm). Corresponding regions (about 700 μm long) from the naïve nerves are shown. The bar represents 50μm. **(B)** Quantitative analysis of the intensity of mu-receptor staining (expressed as the number of positively -stained pixels) in the sciatic nerves of naïve, sham-operated and CCI animals at 2 days and at 2 weeks after surgeries. **(C)** Quantitative analysis showing the number of mu-receptor-expressing fibers in the sciatic nerves of naïve, sham-operated and CCI animals at 2 days and at 2 weeks after surgeries. In B and C the data are expressed as mean ± SEM. N = 5-6 mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way RM ANOVA and Bonferroni t-test or one-way ANOVA on ranks). Ipsi, ipsilateral; contra, contralateral; prox, proximal; farprox, far proximal; n.d., not determined.

Delta-receptor immunoreactivity was enhanced directly proximal to the most proximal ligature, both at 2 days and at 2 weeks following CCI (Fig. 4.10 A). Quantitative analysis of an area of approximately 0.2 mm^2 revealed a robust and significantly higher intensity of delta-receptor staining proximally to the ligature as compared to areas located far proximally and distally to the CCI, and to the nerves of naïve and sham-operated mice at 2 days ($p < 0.001$, one-way RM ANOVA and Bonferroni t-test, Fig. 4.10 B). At 2 weeks after nerve injury, the increase in delta-receptor immunoreactivity proximal to the ligature was lower when compared to the corresponding increase at 2 days ($p < 0.001$, t-test, Fig. 4.10 B), but the staining intensity was still significantly higher compared to the intensity of staining in naïve animals ($p < 0.05$), in the ipsilateral and contralateral nerves of sham-operated animals ($p < 0.001$), in the sites far proximal ($p < 0.05$) and distal ($p < 0.001$) to the ligatures and in the contralateral nerves of CCI animals ($p < 0.05$, one-way RM ANOVA and Bonferroni t-test; Fig. 4.10 B).

The number of delta-positive fibers in naïve, sham-operated, and CCI sciatic nerves (Fig. 4.10 C) was also assessed within the same area. At 2 days, the number of delta-receptor immunoreactive fibers was increased at the site further proximal to the most proximal ligature compared to all other conditions ($p < 0.001$, one-way RM ANOVA and Bonferroni t-test). At 2 weeks, the mean number of delta-receptor-positive fibers at the further proximal site was significantly higher than the number of fibers in naïve nerves ($p < 0.05$), in the ipsilateral and contralateral nerves of sham-operated animals ($p < 0.05$) and distally in the injured nerves ($p < 0.001$), but was not significantly different compared to the contralateral nerves of CCI animals ($p > 0.05$, one-way RM ANOVA and Bonferroni t-test; Fig. 4.10 C). The number of fibers at the site far proximal to ligation was decreased at 2 weeks when compared with 2 days ($p < 0.05$, t-test, Fig. 4.10 C).
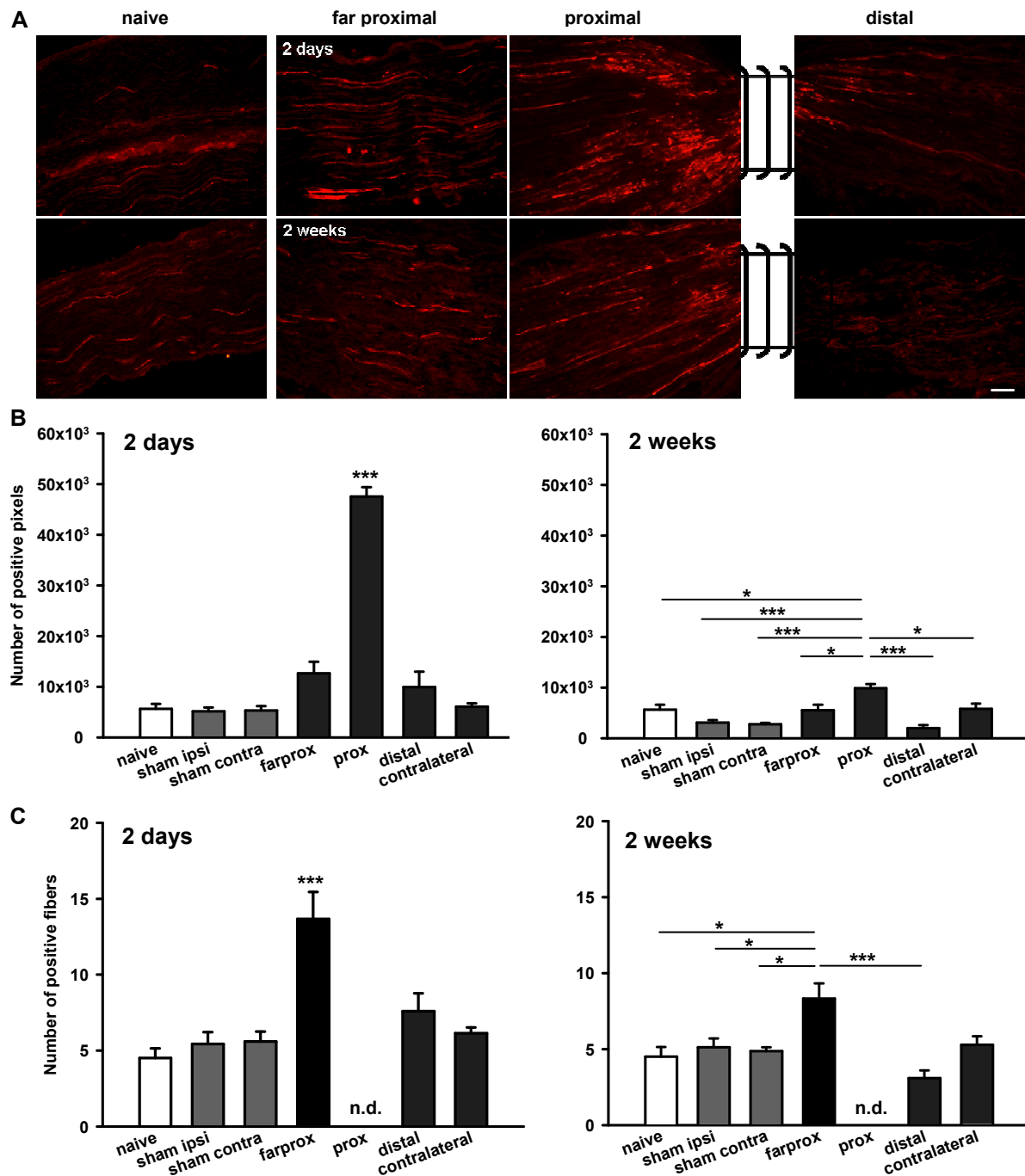
Figure. 4.10: Expression of delta-receptors in the sciatic nerves.
(A) Representative immunofluorescence images showing delta-receptor expression in the sciatic nerves of naïve mice and in the injured nerves at 2 days and 2 weeks following CCI. The images show areas immediately proximal (about 0-700 μm) and further proximal (about 700-1400 μm) to the most proximal ligature, as well as the area immediately distal to the most distal ligature (about 0-700 μm). Corresponding regions (about 700 μm long) from naïve nerves are shown. The bar represents 50 μm.
(B) Quantitative analysis of the intensity of delta-receptor staining (expressed as the number of positively-stained pixels) in the sciatic nerves of naïve, sham-operated and CCI animals at 2 days and at 2 weeks after surgeries. **(C)** Quantitative analysis showing the number of delta-receptor expressing fibers in the sciatic nerves of naïve, sham-operated and CCI animals at 2 days and at 2 weeks after surgeries. In B and C the data are expressed as mean ± SEM. N = 5-6 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001 (one-way RM ANOVA and Bonferroni t-test). Ipsi, ipsilateral; contra, contralateral; farprox, further proximal; prox, proximal; n.d., not determined.

4.3.3. Mu- and delta-receptor expression in the hind paws

In the hind paw skin from naïve and CCI-operated animals, mu-receptors were found predominantly in nerve fibers within the dermis, which sometimes extended into the epidermis (Fig. 4.11 A). In the paw skin of untreated animals the mean number of fibers expressing mu-receptors within an area of approximately 0.19 mm^2 was 4.5 and this number did not significantly change at 2 days or 2 weeks after CCI or sham surgery (p > 0.05, one way RM ANOVA; Fig. 4.11 B).

Figure. 4.11: Expression of mu-receptors in the hind paw tissue following sciatic nerve injury.
(A) Representative immunofluorescence images showing mu-receptor expressing fibers (marked with arrows) in paw tissues from naïve animals and in the ipsilateral paw tissue from CCI animals at 2 days and 2 weeks following CCI. The bar represents 50 μm. E, epidermis; D, dermis.
(B) Quantitative analysis of mu-receptor expressing fibers in the paw tissue of naïve, sham-operated and CCI animals at 2 days and at 2 weeks after surgeries. Data are expressed as mean ± SEM. N = 5-6 mice per group. There were no statistically significant differences among treatments (p > 0.05, one-way RM ANOVA). Ipsi, ipsilateral; contra, contralateral.

Similar to mu-receptors, delta-receptors were found predominantly in sensory nerve fibers within the dermis and occasionally in the epidermis in the hind paws of naïve and CCI animals (Fig. 4.12 A). In contrast to mu-receptors, the mean number of fibers expressing delta-receptors within an area of approximately 0.22 mm^2 varied between the paw tissues of

naïve, sham-operated and CCI animals (Fig. 4.12 B). At 2 days after nerve injury, the paw tissue ipsilateral to the CCI exhibited a significantly lower number of delta-receptor positive fibers when compared to the contralateral side of CCI animals and to naïve animals ($p < 0.05$, one-way RM ANOVA and Bonferroni t-test; Fig. 4.12 B). However, there were no significant differences between the ipsilateral CCI side and sham-operated animals ($p > 0.05$, one-way RM ANOVA; Fig. 4.12 B). At 2 weeks, the number of delta-receptor expressing fibers in the ipsilateral paw tissue of CCI animals was significantly decreased when compared to all other conditions, including paws of naïve animals ($p < 0.01$), ipsilateral and contralateral paws of sham-operated animals ($p < 0.001$ and $p < 0.05$, respectively) and the contralateral paws of CCI animals ($p < 0.05$, one-way RM ANOVA and Bonferroni t-test; Fig. 4.12 B). There was no difference in the decrease at the ipsilateral side of injured animals at 2 days compared to 2 weeks ($p > 0.05$, t-test).
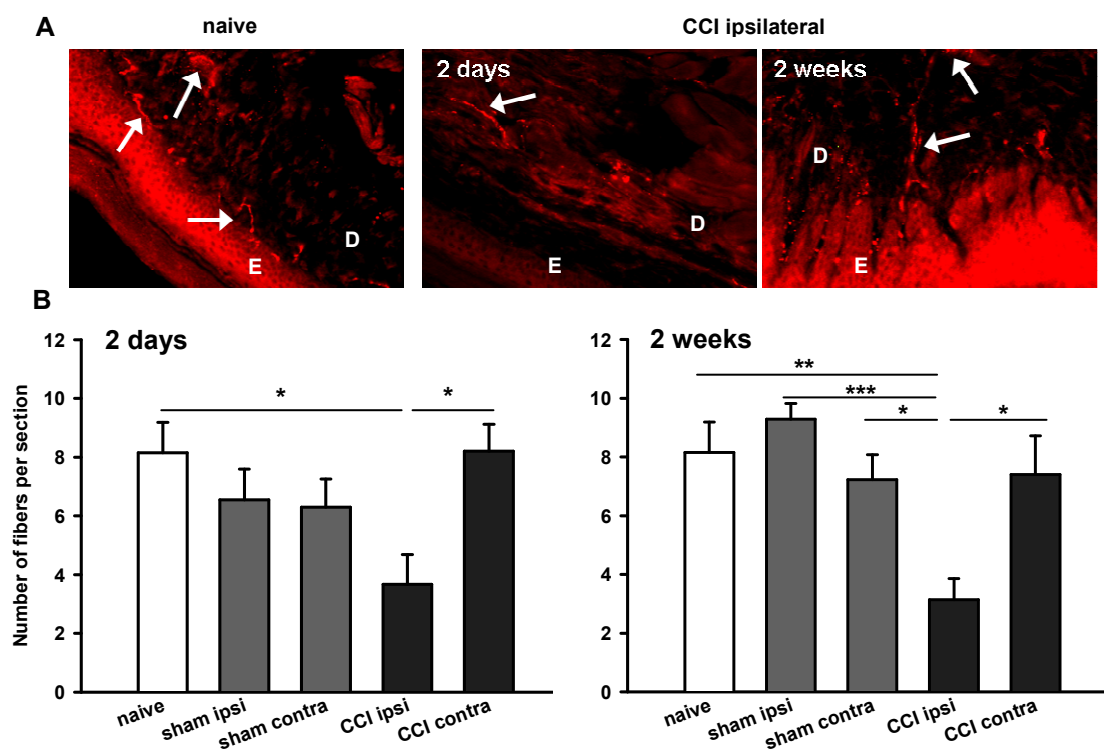
Figure. 4.12: Expression of delta-receptors in the hind paw tissue following sciatic nerve injury.
(A) Representative immunofluorescence images showing delta-receptor expressing fibers (marked with arrows) in paw tissues from naïve animals and in the ipsilateral paw tissue from CCI animals at 2 days and 2 weeks following CCI. The bar represents 50 μm. E, epidermis; D, dermis.
(B) Quantitative analysis of delta-receptor expressing fibers in the paw tissue of naïve, sham-operated and CCI animals at 2 days and at 2 weeks after surgeries. Data are expressed as mean ± SEM. N = 5-6 mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way RM ANOVA and Bonferroni test). Ipsi, ipsilateral; contra, contralateral.

4.4. Effects of mu-opioid receptor agonist on sensory fiber function in neuropathic pain

The studies described under paragraphs 4.1 and 4.3 revealed that under neuropathic pain conditions, opioid receptors are expressed in sensory neurons in the DRG, in the trunk of the injured sciatic nerve as well as in its terminal branches innervating the hind paw. The next goal was to verify the functional relevance of these receptors. For this purpose, we investigated mu-receptors on peripheral terminals of sensory fibers using electrophysiological in vitro skin-nerve method. The saphenous nerve (the largest branch of the femoral nerve) can be conveniently isolated and is commonly used in this method (Koltzenburg et al., 1997; Reeh, 1986; Zimmermann et al., 2009). In addition, it contains exclusively sensory fibers, which prevents possible motor neuron interference during the recordings. We adopted the CCI of the saphenous nerve (Walczak et al., 2006) and used it in our recordings in comparison with non-operated and sham-operated nerves. Specifically, we examined whether a CCI of the saphenous nerve leads to enhanced mechanical sensitivity in vivo, whether electrophysiological properties of sensory fibers change upon nerve injury and whether they can be modulated by the mu-opioid receptor agonist DAMGO applied to the sensory fiber receptive fields in the skin. Initial experiments confirmed a notion that has been already reported for in vitro skin-nerve measurements at one day following a CCI (Walczak et al., 2006): 2 days after the operation of the saphenous nerve it was hardly possible to record action potentials evoked by stimulation of the hind paw skin from the isolated nerve, whereas recordings were possible at 2 weeks after injury. Therefore, experiments were performed at 2 weeks after CCI only. Moreover, the fiber loss seemed to be related mainly to Aβ fibers, which was reflected in the overall percentage of fibers that we were able to analyze in injured versus uninjured nerves: Aβ (26% vs. 45%), Aδ (53% vs. 43%), C fibers (21% vs. 13%). A predominant loss of Aβ fibers following a CCI is in line with earlier studies (Kajander and Bennett, 1992) and likely made it easier to identify Aδ and C fibers in injured nerves. Spontaneous activity was noticed in a low number of fibers from injured nerves; however, it was seldom possible to localize a respective cutaneous receptive field, assuming that most spontaneous activity did not originate at the peripheral nerve endings.

4.4.1. Mechanical hypersensitivity after a CCI of the saphenous nerve

Unilateral CCI of the saphenous nerve resulted in a profound mechanical hypersensitivity manifested by significantly lower thresholds to von Frey hairs in the hind paws innervated by the injured nerves at 2 weeks following CCI compared to the thresholds before CCI and to the thresholds in the contalateral paw of CCI mice and in both hind paws of sham-operated animals ($p < 0.001$, two-way RM ANOVA and Bonferroni test, Fig. 4.13). This experiment was performed by a member of our group, Dr. Dominika Labuz.
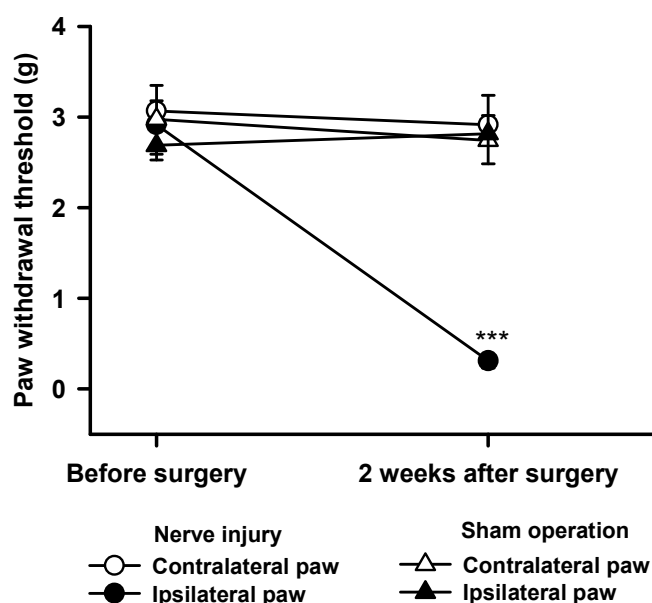
Figure 4.13: Mechanical hypersensitivity to von Frey filaments following CCI of the saphenous nerve. Measurements were performed before (baseline) and 2 weeks after CCI. Data are expressed as means ± SEM. N = 6 mice per condition. *** $p < 0.001$, (two-way RM ANOVA and Bonferroni test).

4.4.2. Electrophysiological responses of sensory fibers in non-operated and sham-operated nerves

First, we analyzed whether a sham operation of the saphenous nerve (n = 8) induced changes in the conduction velocity or the mechanical von Frey threshold of sensory fiber types. There were neither significant differences in the conduction velocity of Aδ ($p > 0.05$, t-test), Aβ or C fibers ($p > 0.05$, Mann-Whitney test) between non-operated and sham-operated nerves, nor between the baseline mechanical thresholds of corresponding fiber types ($p > 0.05$, Mann-Whitney Test, Table 4.1).

Table 4.1: Baseline conduction velocities and mechanical thresholds of sensory fibers in non-operated and sham-operated nerves

Fiber type	Non-operated nerves		Sham-operated nerves	
	Conduction velocity	Threshold	Conduction velocity	Threshold
Aβ	14.6 ± 0.7 m/s	0.14 ± 0.02 g	15.7 ± 0.9 m/s	0.16 ± 0.04 g
Aδ	5.9 ± 0.5 m/s	0.34 ± 0,05 g	5.7 ± 0.6 m/s	0.23 ± 0.08 g
C	0.7 ± 0.1 m/s	0.63 ± 0.14 g	0.6 ± 0.1 m/s	0.6 ± 0.1 g

The conduction velocity is expressed in meter per second, the von Frey threshold in grams.Data are presented as means ± SEM. N = 5 - 15 tested fibers per nerve type. There were no significant differences between the conduction velocities or the baseline thresholds of non-operated and sham-operated nerves or ($p > 0.05$, t-test or Mann-Whitney Test).

We also assessed whether a sham surgery affected the latency to response or the discharge rate to increasing mechanical stimulation. No significant differences were found in the baseline values for the latency of all fiber types or the discharge rates for all displacements and fiber types between the non-operated and sham-operated nerves ($p > 0.05$, two-way RM ANOVA, data not shown). As an example, the mean number of action potentials of each fiber type from the last displacement (768 μm) of unligated and sham-operated nerves was calculated and is presented in table 4.2. In response to all results, the data from non-operated and sham-operated nerves were combined and served as control data from uninjured nerves in comparison to injured (CCI-ligated) nerves.

Table 4.2: Baseline discharge rates to the highest displacement of nanomotor-induced mechanical stimulation in sensory fibers of non- and sham-operated nerves

Fiber type	Non-operated nerves	Sham-operated nerves
RAM	4.7 ± 0,8	3.8 ± 1
SAM	64.8 ± 10.6	76.6 ± 14.1
D-hairs	14.6 ± 2.9	15.5 ± 4.5
AM	129.7 ± 14.6	97.5 ± 13.8
C	91 ± 26.6	66.8 ± 25.8

The mean number of action potentials during a 10 s stimulation to the last displacement (768 μm) is presented. Data are expressed as means ± SEM. N = 5 - 15 tested fibers per nerve type. No statistically significant differences were found between non-operated and sham-operated nerves for each fiber type ($p > 0.05$, t-test).

4.4.3. Conduction velocity and mechanical threshold to von Frey stimulation in sensory fibers of injured and uninjured nerves

Figure 4.14 shows the baseline physiological properties of single sensory fibers from uninjured and injured saphenous nerves. First, fibers were classified based on their conduction velocity, according to Koltzenburg et al. (1997). The conduction velocity was calculated as the distance from the receptive field to the recording electrode (in millimeters) divided by the electrical latency of the action potential. Fibers conducting > 10 m/s were assigned to Aβ, those conducting 1.2 – 10 m/s as Aδ and those with conduction velocity < 1.2 m/s were classified as C fibers. The mean conduction velocity of Aβ fibers from uninjured nerves was 15 ± 0.4 m/sec, of Aδ 5.4 ± 0.3 m/sec and of C fibers 0.7 ± 0.08 m/sec. The mean conduction velocities of the respective fiber types after the CCI-operation were significantly different for Aβ (13.5 ± 0.5 m/sec) and Aδ (4.5 ± 0.2 m/sec) ($p > 0.05$, Mann-Whitney Test, Fig. 2 A), however the decrease was very small in amplitude. C fibers revealed no difference in their conduction velocity after injury. Then, mechanical thresholds of each fiber type were tested with von Frey filaments applied to the fibers' receptive fields. In uninjured nerves, the mechanical threshold was the lowest for Aβ fibers (0.14 ± 0.02 g), increased for Aδ (0.34 ± 0.05 g) and was the highest for C fibers (0.63 ± 0.14 g) (Fig. 4.14 B). The data on threshold values were not further subclassified into RAM, SAM, D-hair, AM and C fibers, as not all fibers evaluated for their von Frey mechanical threshold could be additionally tested for their discharge pattern with the nanomotor. However, those Aβ fibers that were classified as RAM (0,09 ± 0,02 g) or SAM (0,16 ± 0,03 g) based on the data from nanomotor stimulation revealed a similar threshold, whereas Aδ fibers classified as D-hairs had a considerably low threshold (0.02 ± 0.005 g) and the threshold for AM fibers (0.51 ± 0.06 g) was in the range of C fibers. These relationships of Aβ and Aδ subtypes are similar to previous studies (Koltzenburg et al., 1997; Milenkovic et al., 2008). There were no significant differences in the von Frey mechanical thresholds of the respective fiber types (Aβ, Aδ or C fibers) before and after nerve injury ($p > 0.05$, Mann-Whitney Test; Fig. 4.14 B). Also, this condition did not significantly alter the thresholds of Aβ and Aδ fiber subpopulations ($p > 0.05$, T-test or Mann-Whitney test, data not shown). Together, these data indicate that a nerve injury has some effect on the conduction velocity, but none on the mechanical threshold of primary sensory afferent fibers.
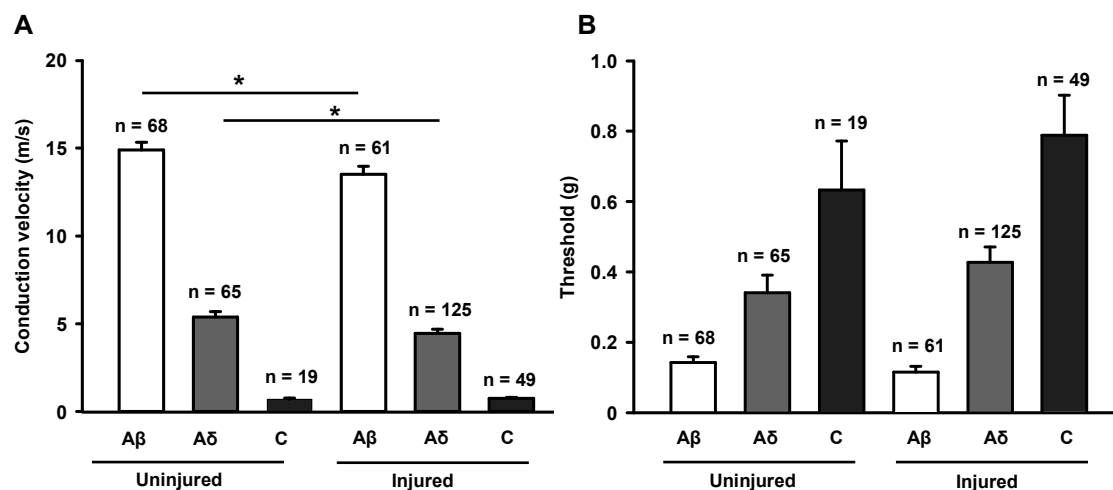
Figure 4.14: Conduction velocity and mechanical threshold of sensory fibers from uninjured and injured nerves. (A) Mean conduction velocity in m/s and **(B)** mean mechanical threshold to von Frey hair stimulation in grams of Aβ, Aδ and C fibers of uninjured and injured nerves at 2 weeks after CCI. Data are expressed as means ± SEM. N = number of tested fibers per group. Significant differences between respective (e.g. C-C) fibers of uninjured versus injured nerves are indicated. *$p < 0.05$ (Mann-Whitney Test).

4.4.4. Mechanical latency to constant mechanical stimulation of different intensity in sensory fibers of uninjured and injured nerves

Mechanoreceptors vary in persistence of their responses to constant mechanical stimulation. A computer-controlled nanomotor was used to apply mechanical stimulation of increasing strength (by logarithmically increasing the displacement in µm), each with a constant (10 sec) duration. The discharge pattern was used to differentiate between rapidly- and slowly-adapting fibers and to calculate the discharge rate and latency of response to increasing mechanical stimulation. The latter can be measured by detecting the time difference between the onset of the mechanical stimulus and the onset of the first action potential subtracted by the electrical latency of the fiber (Milenkovic et al., 2008). The relationship between latency and mechanical stimulus strength was very characteristic for fibers types. Measurements in uninjured and injured nerves revealed that the shortest latencies for rapidly-adapting RAM (7.4 ± 1.2 ms at displacement 20) and D-hair fibers (9 ± 3.7 ms at displacement 10) were found within the lowest mechanical stimuli used. Interestingly, the mechanical latencies in fibers with a slowly adapting response (SAM, AM and C-fibers) were different compared to fibers with a rapidly adapting response. Being initially very long, the mechanical latencies of SAM, AM and C fiber shortened to a plateau value as stimulus strength increased and therefore revealed the shortest latencies at higher displacements. Still, the minimum mean mechanical latency observed for C-fibers (143.2 ± 86 ms at step 320) was

much longer than that found for AM fibers (29 ± 3 ms at step 160) and for SAM fibers (26 ± 4.5 ms at step 160). The nerve injury did not significantly change the latency to mechanical stimulation in any of the sensory fiber types ($p > 0.05$, two-way RM ANOVA; Fig. 4.15).
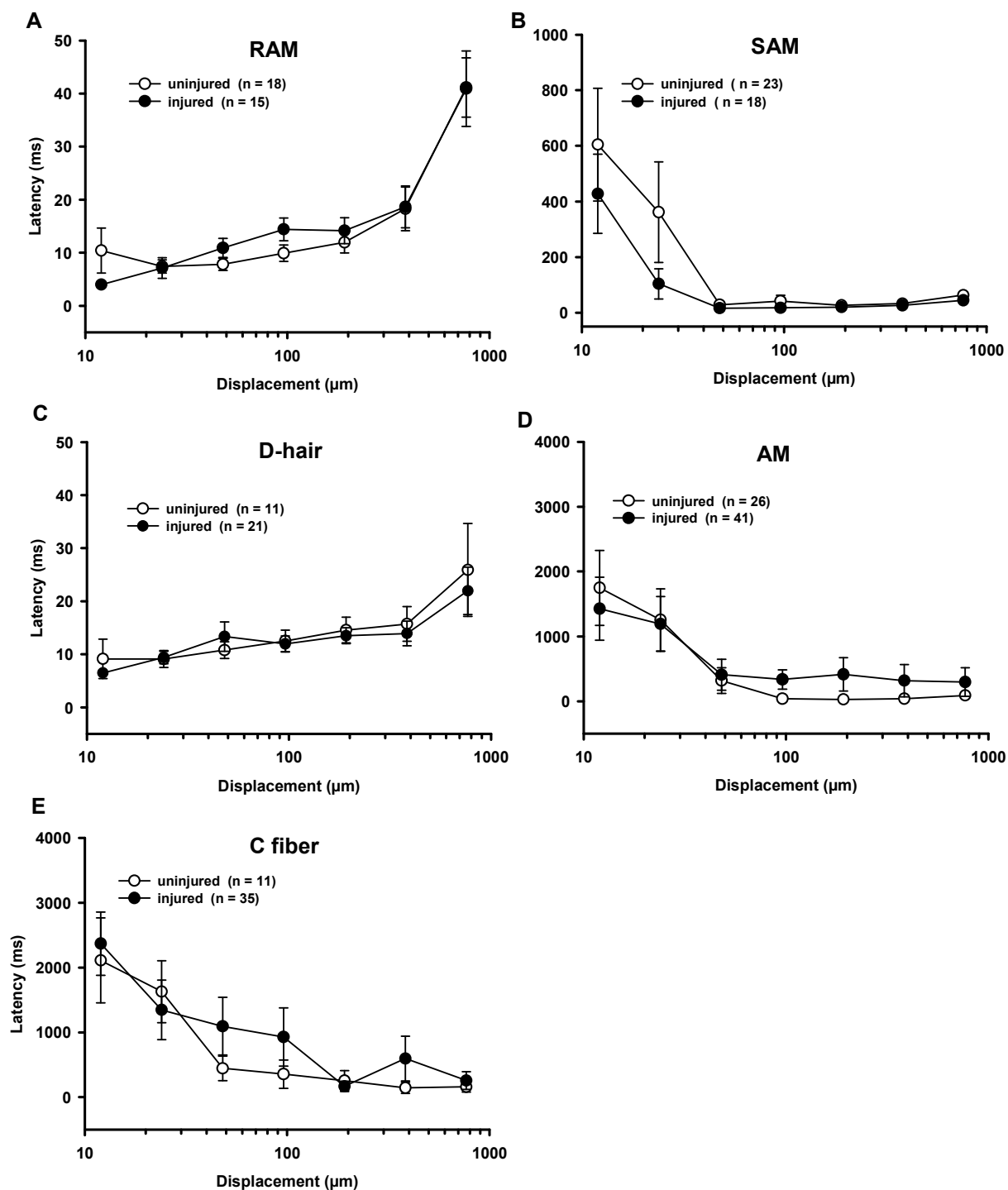
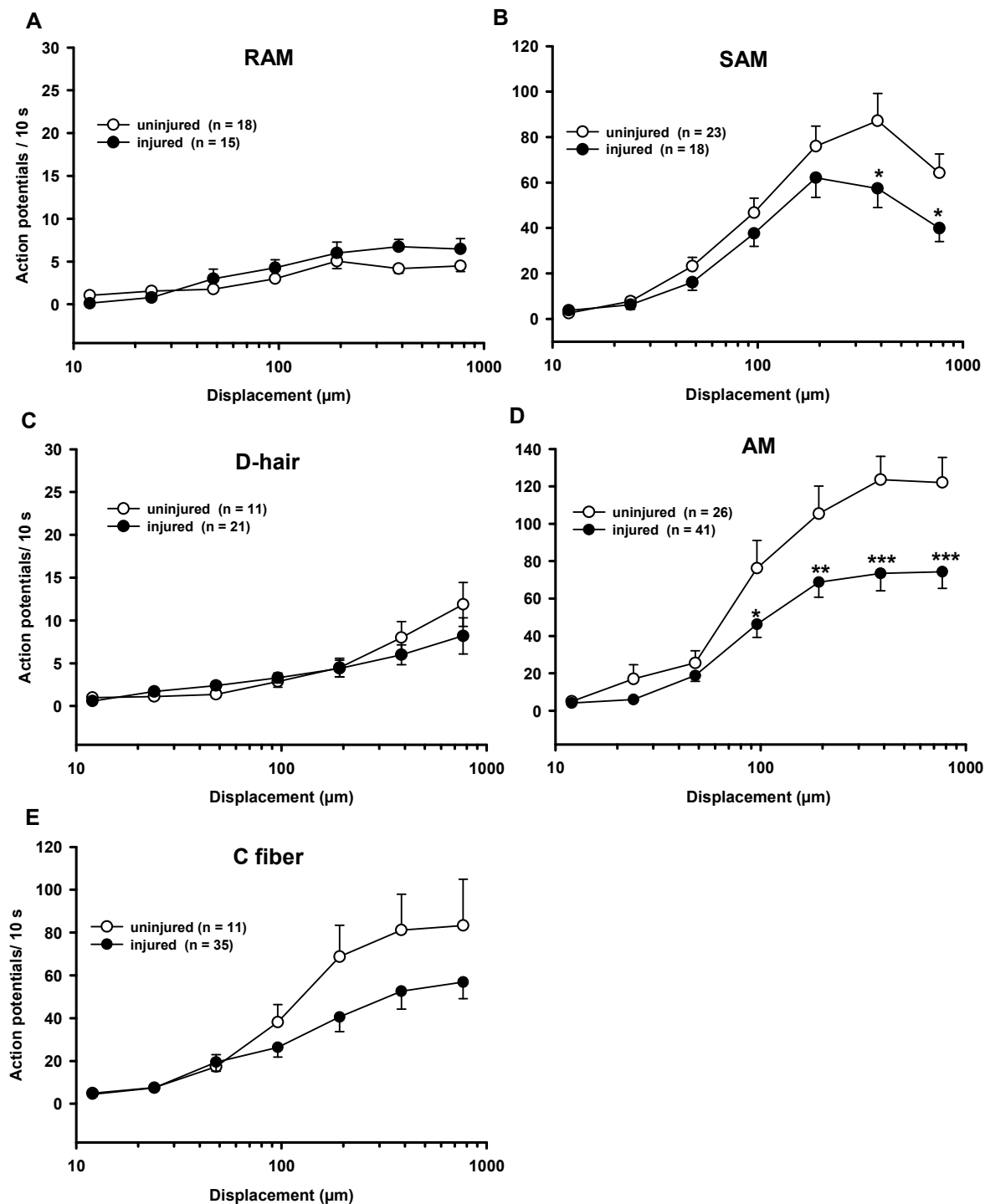**Figure 4.15: Baseline mechanical latency of sensory fibers from uninjured and injured nerves.** (A-E) Latency of response to a 10 s stimulation at increasing displacements in RA, SA, D-hairs, AM and C fibers from uninjured and injured nerves. Data are expressed as means $\pm$ SEM. N = fibers per nerve type. There were no significant differences between uninjured and injured nerves (two-way RM ANOVA).

4.4.5. Discharge rates to constant mechanical stimulation of increasing intensities in sensory fibers of uninjured and injured nerves

The discharge rate is plotted as the number of all action potentials evoked by a constant mechanical stimulus of a defined duration (10 sec) and a specific strength. All fiber types revealed significant differences with respect to the discharge rate in response to displacements of different intensities in uninjured and injured nerves ($p < 0.001$, two-way RM ANOVA and Bonferroni t-test, data not shown), indicating that the discharge rate depended on the strength of mechanical stimulation. However, we noticed that this effect was most prominent in slowly-adapting AM, SAM and C fibers, as it affected more displacements. Moreover, AM receptors demonstrated the highest mean discharge rates, consistent with the findings of other groups (Milenkovic et al., 2008; Slugg et al., 2000).

Interestingly, quantitative analysis revealed that the discharge rate was attenuated in slowly-adapting SAM and AM fibers in injured compared to uninjured nerves (Fig. 4.16). Thus, the discharge rate in injured nerves significantly decreased in SAM ($p < 0.05$ at displacements 320 and 640) and in AM fibers ($p < 0.05$ at displacement 80; $p < 0.01$ at displacement 160; and $p < 0.001$ at displacements 320 and 640) (two-way RM ANOVA and Bonferroni t-test; Fig. 4.16). The action potential frequency tended to decrease in C fibers in injured nerves, although the effect was not significantly different from non-injured nerves ($p > 0.05$, two-way RM ANOVA; Fig. 4.16). There were also no significant differences in case of rapidly-adapting RAM and D-hairs ($p > 0.05$, two-way RM ANOVA; Fig. 4.16).

Figure 4.16: Baseline discharge rates of sensory fibers from uninjured and injured nerves.
(A-E) Number of action potentials during a 10 s stimulation at increasing displacement of RAM, SAM, D-hairs, AM and C fibers from uninjured and injured nerves. Data are expressed as means ± SEM. N indicates the number of tested fibers per nerve type. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to the respective value of uninjured nerves (two-way RM ANOVA and Bonferroni t-test).

4.4.6. Effects of DAMGO on the mechanical threshold of sensory fibers in uninjured and injured nerves

Next, we were interested whether the mu-receptor agonist DAMGO, applied to the receptive fields of sensory fibers, can modify their von Frey hair-induced mechanical threshold and whether its effects differ between injured and uninjured nerves. If DAMGO increased a fiber's von Frey threshold, the fiber was defined as DAMGO-responsive. The threshold was considered elevated if following DAMGO application, a higher von Frey hair force than at the baseline was necessary to elicit at least one action potential. Fibers which did not meet these criteria were considered DAMGO-non-responsive. DAMGO never decreased fibers' thresholds. We applied DAMGO in a dose of 100 µM, as this dose proved most effective in our pilot experiments testing doses in a range of 1-500 µM.

In uninjured nerves, virtually none Aβ (3%) and very few Aδ (~ 6%) and C fibers (~15%) were DAMGO-sensitive (Fig. 4.17 A). In sharp contrast, about 30% of Aδ and about 45% of C fibers, but none of the Aβ fibers, were DAMGO-responsive in injured nerves (Fig. 4.17 B). Therefore, the specificity of the effects of DAMGO on von Frey thresholds was investigated in DAMGO-responsive Aδ- and C fibers in injured nerves (Fig. 4.17, C and D). We found that the baseline von Frey thresholds of Aδ and C fibers were significantly elevated by DAMGO (100 µM), which was reversed by a washout of DAMGO or by the pretreatment of the receptive fields with 100 µM CTOP (Cys^2-Tyr^3-Orn^5-Pen^7-amide), a mu-receptor antagonist ($p < 0.05$, one-way RM ANOVA on ranks and Tukey test, C and D). All Aδ fibers that were considered DAMGO-responsive had high baseline mechanical thresholds (mean threshold: 0.4 ± 0.06 g) and those tested with the nanomotor were slowly-adapting, indicating that all DAMGO-responsive Aδ fibers most likely were AM nociceptors. In line with it, no classified D-hair fiber revealed an elevated von Frey hair threshold after DAMGO treatment. The number of Aδ and C fibers tested after washout of DAMGO or after pretreatment with CTOP is lower than the numbers for baseline and post-DAMGO measurements, since not all fibers responded to the mechanical stimulation throughout the whole testing procedure.

The application of control buffer to the receptive field of fibers served as an additional control for a DAMGO-specific effect. There was no significant difference between the baseline mechanical threshold of Aβ, Aδ or C fibers and their respective mechanical threshold after application of control buffer ($p > 0.05$, one-way RM ANOVA; table 4.3). The results of the pretreatment with CTOP and the application of buffer point to a mu-receptor mediated effect in C and Aδ (AM) fibers.
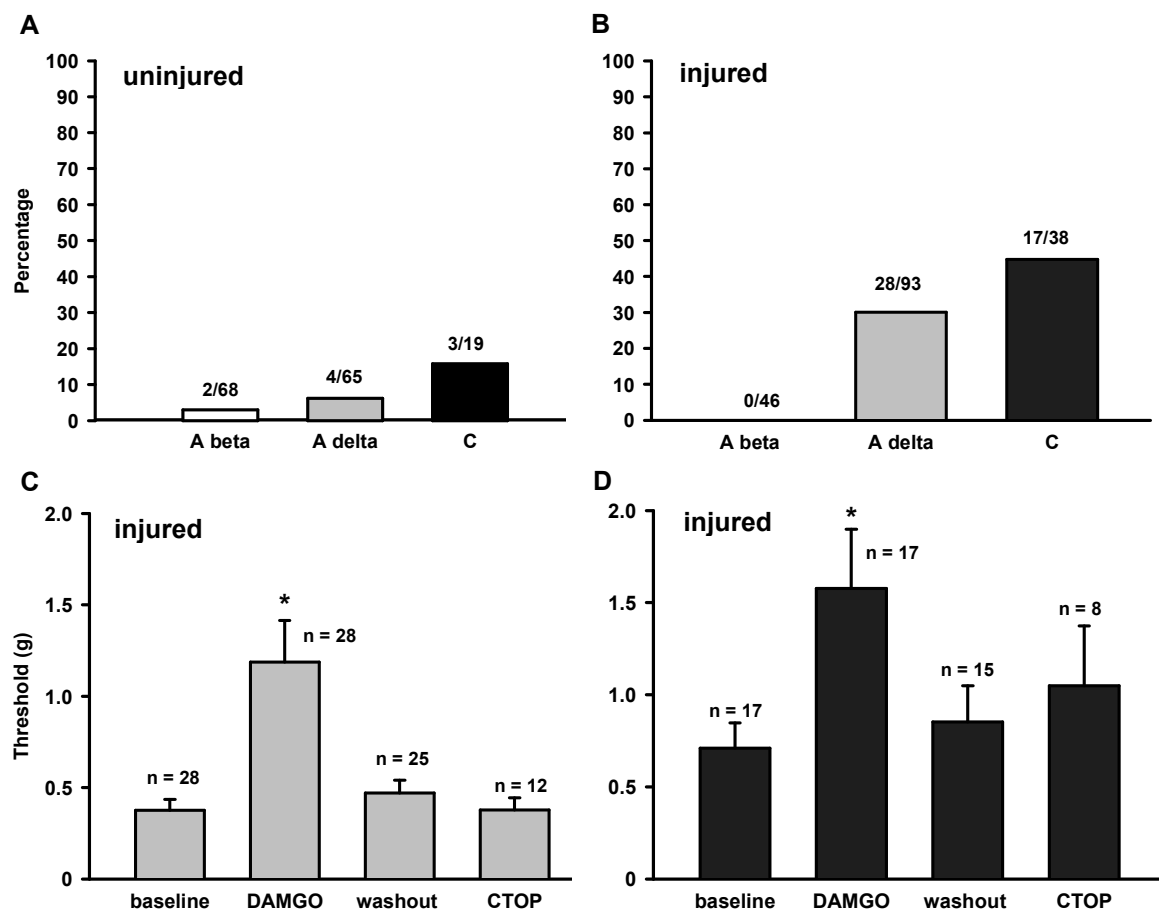
Figure 4.17: Effect of DAMGO on von Frey mechanical thresholds of sensory fibers in uninjured and injured nerves.
(A, B) Percentage of Aβ, Aδ and C fibers that showed an increase in the threshold after DAMGO application to their receptive fields in uninjured (A) and injured nerves (B). The numbers above the bars represent the number of DAMGO-responsive fibers per the total number of tested fibers.
(C, D) Mechanical thresholds before (baseline) and after DAMGO application, after DAMGO washout and after CTOP and DAMGO application in DAMGO-responsive Aδ fibers (C) and C fibers (D) in injured nerves. Data are expressed as means ± SEM. N indicate the number of DAMGO-responsive fibers tested per condition. *$p < 0.05$ (one-way RM ANOVA on ranks and Tukey test).

Table 4.3: Mechanical von Frey thresholds of sensory fibers from injured nerves before and after application of control buffer to the fibers' receptive fields

Fiber type	Baseline	Control buffer
Aβ (15)	0.12 ± 0.03 g	0.12 ± 0.03 g
Aδ (32)	0.45 ± 0.09 g	0.46 ± 0.09 g
C (11)	0.79 ± 0.2 g	0.8 ± 0.19 g

The data represent the von Frey thresholds in grams and are expressed as means ± SEM. The number of tested fibers is depicted in brackets next to each fiber type. There were no statistically significant differences between the baseline thresholds and those after buffer application for each fiber type ($p > 0.05$, Wilcoxon Test).

4.4.7. Effects of DAMGO on the latency of response of sensory fibers in uninjured and injured nerves

The nanomotor protocol was run before and after the application of DAMGO and, if possible, also after the 15-min-washout of DAMGO. The question addressed by this protocol was, whether the latency and the number of action potentials (discharge rates) per each displacement applied to the receptive fields of RAM, SAM, D-hairs, AM and C fibers change after the application of DAMGO, and whether it matters, if the nerve is injured or not. As described above, AM and C fibers in injured nerves comprised DAMGO-responsive and DAMGO-non-responsive fibers (based on DAMGO-induced elevations of von Frey mechanical thresholds). To find out whether the effects of DAMGO on mechanical latencies and discharge rates of these fibers correlate with the effects on mechanical thresholds, we analyzed the data from DAMGO-responsive and DAMGO-non-responsive AM and C fibers together and separately. This was not done in case of Aβ fibers (RAM and SAM) and the D-hairs of Aδ fibers, because all were DAMGO-non-responsive (see 4.4.6). No significant differences were found in the latency of response to nanomotor mechanical stimulation following DAMGO application to the receptive fields of RAM, SAM, D-hairs, AM and C fibers from uninjured (data not shown) and injured nerves (Fig. 4.18) ($p > 0.05$, two-way RM ANOVA). The pre- and post-DAMGO values of DAMGO-responsive AM and C fibers of injured nerves were also not significantly different ($p > 0.05$, two-way RM ANOVA, data not shown).
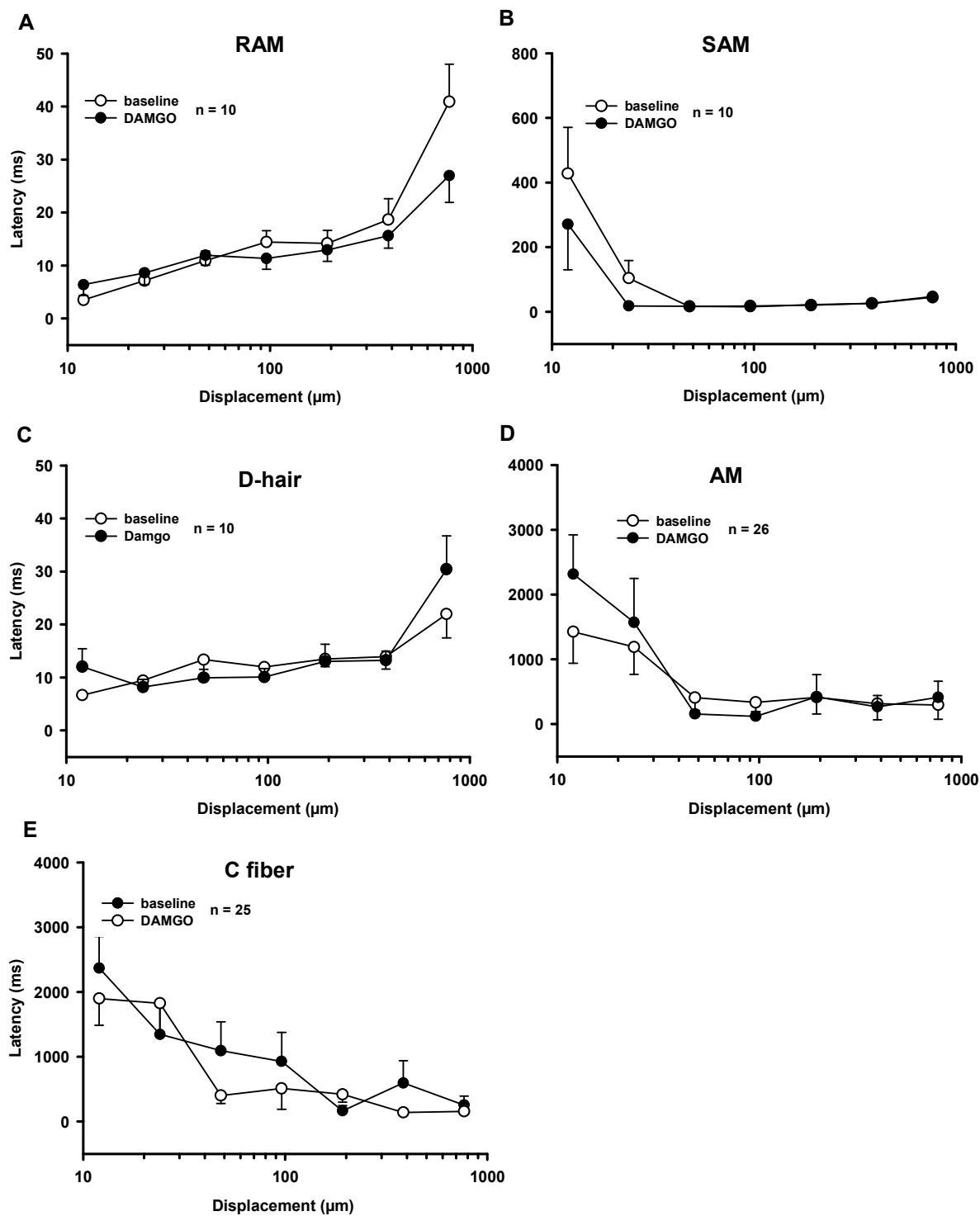
Figure 4.18: Effects of DAMGO on the mechanical latency of sensory fibers in injured nerves.
(A-E) Latency of response to a 10 s stimulation at increasing displacements of RAM, SAM, D-hairs, AM and C fibers of injured nerves at baseline and after DAMGO treatment. Data are expressed as means ± SEM. N indicate the number of tested fibers. No statistically significant differences were found ($p > 0.05$, two-way RM ANOVA).

4.4.8. Effects of DAMGO on discharge rates of sensory fibers in uninjured versus injured nerves

The discharge rates of RAM, SAM, D-hair, AM and C fibers in uninjured nerves at baseline levels were similar to those in uninjured nerves shown in Figure 4.16. DAMGO did not significantly change the discharge rates of RAM, SAM and D-hairs, as well as of AM and C fibers from uninjured nerves, regardless whether all or DAMGO-responsive AM and C fibers were analyzed ($p > 0.05$, two-way RM ANOVA, data not shown). DAMGO did also not change the discharge rates of RAM, SAM and D-hairs from injured nerves ($p > 0.05$, two-way RM ANOVA, Fig. 4.19, A-C). It also did not affect discharge rates of AM fibers when DAMGO-responsive and non-responsive fibers were analyzed together (Figure 4.19, D) ($p > 0.05$). However, C fiber discharge rates to higher displacements were significantly reduced by DAMGO already when DAMGO-responsive and non-responsive fibers were analyzed together ($p < 0.05$ at 192 μm and $p < 0.001$ at 384 μm and 768 μm; Figure 4.19, E).
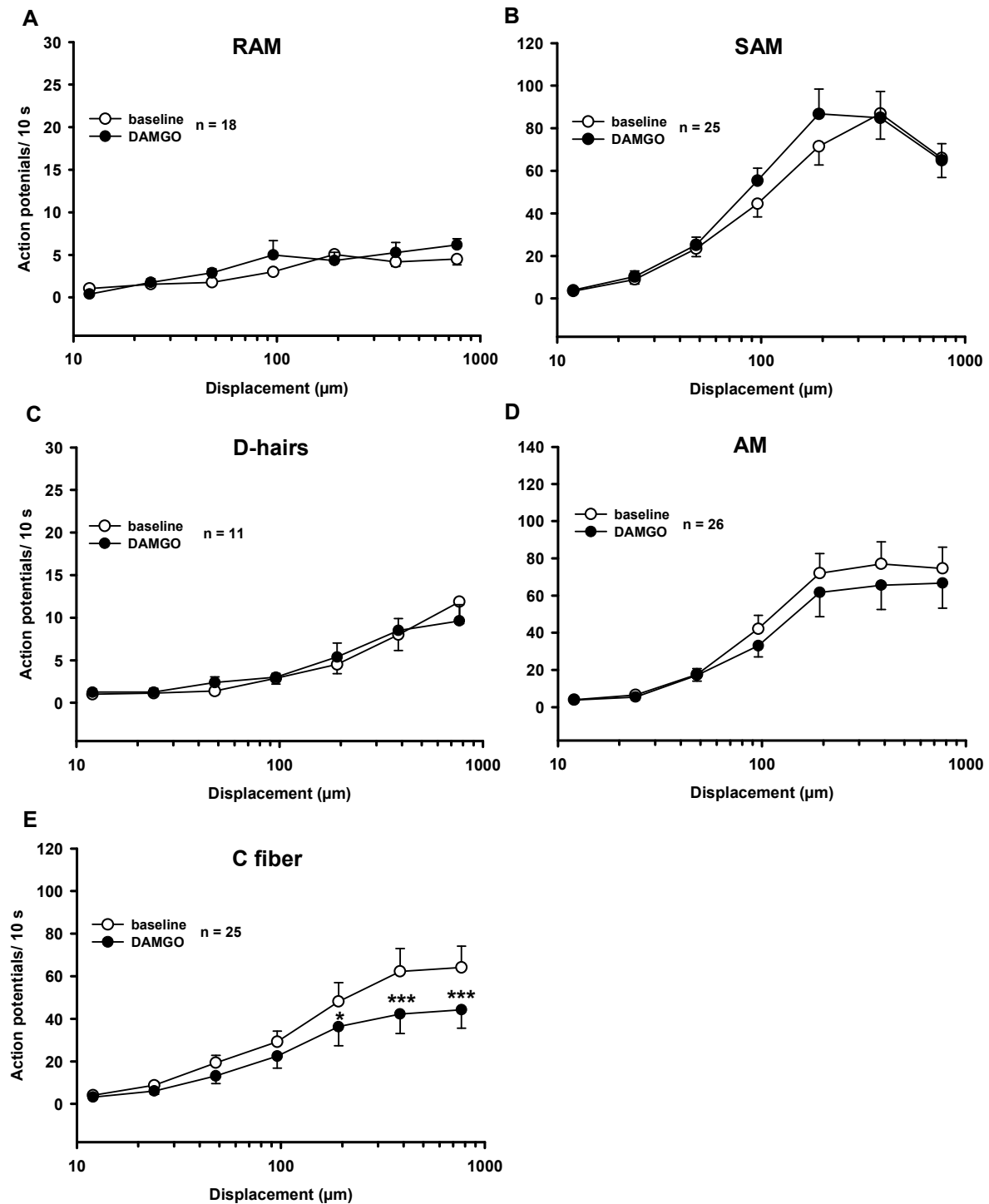
Figure 4.19: Effects of DAMGO on the discharge rates of sensory fibers in injured nerves.
(A-E) Number of action potentials during a 10 s stimulation at increasing displacement of RAM, SAM, D-hairs, AM and C fibers of injured nerves at baseline and after DAMGO treatment. Data are expressed as means ± SEM. N indicates the number of tested fibers per nerve type. *p < 0.05, ***p < 0.001, compared to the respective baseline value (two-way RM ANOVA and Bonferroni t-test).

DAMGO-responsive (with regard to the threshold) AM fibers were evaluated separately and showed a tendency to lowered discharge rates after DAMGO treatment, however there was no significant difference ($p > 0.05$, two-way RM ANOVA, Fig. 4.20, A).

The reduction in the discharge rates of all C fibers was apparently attributed to the DAMGO-responsive (with regard to the threshold) C fibers, as their discharge rates were robustly diminished by DAMGO ($p < 0.05$ at 48 μm and 96 μm $p < 0.01$ at 192 μm and $p < 0.001$ at 384 μm and 768 μm, two-way RM ANOVA, Fig. 4.20, C). DAMGO did not affect DAMGO-non-responsive AM and C fibers in injured nerves ($p > 0.05$, two-way RM ANOVA, data not shown). Control buffer application to the receptive fields of AM and C fibers from injured nerves revealed no significant difference in the fiber discharge between baseline values and post-buffer values (Fig. 4.20, B and D repectively, two-way RM ANOVA, $p > 0.05$).
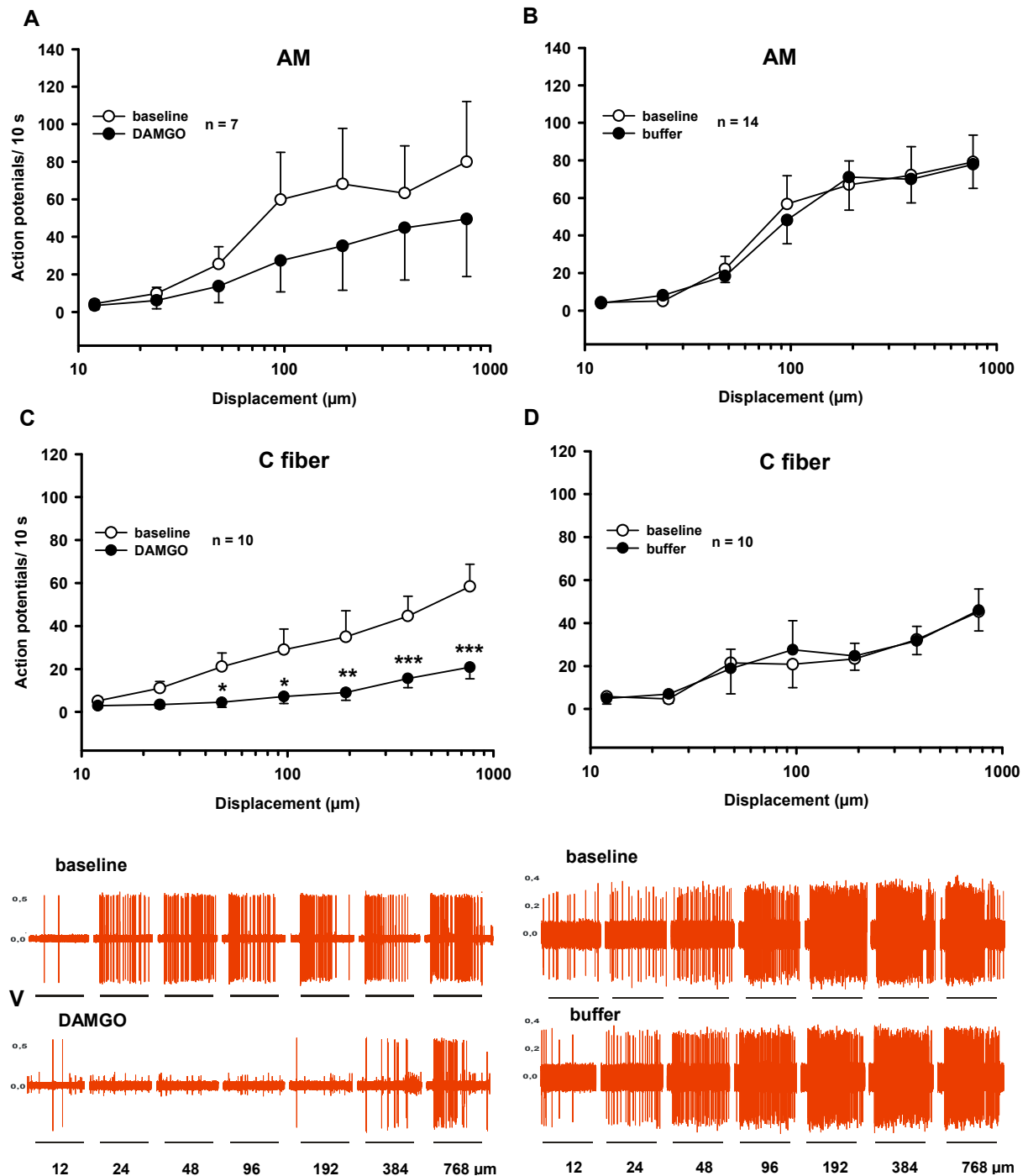
Figure 4.20: Effects of DAMGO on the discharge rates of DAMGO-responsive AM and C fibers and effects of control buffer application in injured nerves.
(A, B) Number of action potentials of AM fibers during a 10 s stimulation at increasing displacement after DAMGO (A) and buffer (B) application.
(C, D) Number of action potentials of C fibers during a 10 sec stimulation at increasing displacement after DAMGO **(C)** and buffer **(D)** application. Lower panels show representative examples of C fiber firing before (baseline) and after DAMGO (C), and before and after control buffer application (D). The displacements are marked with dark lines. The interstimulus sequences are removed. DAMGO and buffer were applied to the fibers' receptive fields. All data are expressed as means ± SEM. N, number of fibers. *p < 0.05, **p < 0.01, ***p < 0.001, compared to baseline (two-way RM ANOVA, Bonferroni t test). V = voltage.

5   Discussion

The present study investigated the impact of a peripheral nerve injury on the expression of opioid receptors in peripheral sensory pathways, and its impact on the thresholds, discharge rates and latencies of primary afferent neurons in response to increasing mechanical stimulation. Moreover, changes of the mechanically evoked responses after activation of mu-receptors by administration of its agonist DAMGO were determined. The major findings of this study are that following a peripheral nerve injury:

- mu- and delta-receptors were mainly localized in peptidergic C and Aδ fibers in peripheral sensory neurons,
- the expression of mu- and delta-receptors in the DRGs was not changed, both receptors were strongly upregulated in the sciatic nerve proximally to the injury, while in the hind paw skin mu-receptor expression was not altered, whereas delta-receptor expression was decreased,
- the thresholds and the latency of action potentials from sensory fibers in response to mechanical stimulation did not vary from uninjured nerves, whereas the conduction velocity of A fibers and the discharge rate of slowly-adapting SA and AM fibers was reduced,
- the activation of mu-receptors on the peripheral terminals of C and Aδ nociceptors increased the thresholds and lowered the discharge rates in response to strong mechanical stimulation of their receptive fields.

5.1.   Differential changes in the expression of peripheral mu- and delta-opioid receptors following nerve injury

5.1.1.   Mu- and delta-receptors are mainly localized in peptidergic C and A fibers following nerve injury

Two days and 2 weeks following nerve injury, we found numerous cells co-expressing mu-or delta-receptors and CGRP, a marker of peptidergic C and A fibers, in the ipsilateral DRGs of sciatic nerves. We could show that positively stained DRG cells were from small to medium in size and only sparsely co-expressed IB4 and NF200. These results suggest that under neuropathic pain conditions, mu- and delta-receptors are expressed in primary sensory peptidergic C and Aδ fibers. The expression pattern of both opioid receptor types is similar to

the one found under naïve conditions: The majority of mu-and delta-receptor protein of naïve mice or rats was found to be expressed in small to medium diameter DRG neurons that also contained CGRP (Dado et al., 1993; Li et al., 1998; Zhang et al., 1998a; Zhang et al., 1998b). In addition, patch-clamp experiments of trigeminal ganglia or DRG cells from naïve animals revealed that potent inhibition of Ca^{2+}-currents by the mu-receptor agonist DAMGO was selective for IB4-negative and mainly small diameter neurons (Taddese et al., 1995; Wu et al., 2004), indicating that these were peptidergic C fibers. However, whereas the expression of mu-receptor protein in small and medium diameter, peptidergic neurons under naïve conditions is mainly accepted, the results of a very recent paper have questioned a similar distribution pattern of the delta-receptor protein.

In their study, Scherrer et al. (2009) used a knockin mouse, in which the endogenous delta-receptor was replaced by a delta-receptor fused to enhanced green fluorescent protein (eGFP). They examined its distribution in sensory neurons of DRGs using immunofluorescence staining with an antibody against GFP. The delta-eGFP receptor was found to be expressed in approximatively 17% of DRG cells whereof most co-stained with NF200 (56%) or co-expressed IB4 (36%). This is in sharp contrast to the abovementioned immunohistochemical results from naïve animals. Scherrer and colleagues tested several commonly used, commercially available delta-receptor antibodies, including one raised against the N-terminus (amino acids 3-17) and the one we used in our study, raised against the first extracellular loop (amino acids 111-120) of the receptor. The authors argued that the antibodies are not able to specifically bind delta-receptors in immunohistochemical preparations, because they immunostained tissue from two delta-receptor knockout mice (Filliol et al., 2000; Zhu et al., 1999). This finding questions the immunohistochemical identification of delta-receptor expression in the present and previous studies.

However, a previous study reported that the N-terminus directed delta-receptor antibody used by Scherrer and colleagues did not stain cultured DRG neurons from the delta-receptor knockout mouse constructed by Filliol et al. (2000), with a disruption of exon 1 (Walwyn et al., 2005). Similarly, brain slices from the delta-receptor knockout mouse constructed by Zhu et al. (1999), with a disruption of exon 2, were shown to be devoid of staining after application of the same delta-receptor antibody (Zhu et al., 1999). Importantly, the N-terminus antibody has been shown to stain identical regions of rat and mouse brain and spinal cord as the antibody against the first extracellular loop used in our study (Dado et al., 1993), suggesting that both indeed recognize delta-receptors.

Furthermore, delta-receptor agonists (DPDPE; met-enkephalin, DTLET) were shown to reduce increases in the extracellular concentrations of SP or CGRP evoked by depolarization of primary sensory neurons *in vitro* (Chang et al., 1989; Jessell and Iversen, 1977; Pohl et al., 1989) or by C-fiber stimulation *in vivo* (Aimone and Yaksh, 1989). Additionally, a direct interaction of delta-receptors with SP (which normally colocalizes with CGRP) (Zhang et al., 1995) has been demonstrated by immunoprecipitation of a tagged delta-receptor with SP in co-transfected cell cultures (Guan et al., 2005). These results strongly suggest a co-expression of delta-receptors with SP and CGRP and consequently an expression in peptidergic C fibers while avoiding the use of any delta-receptor antibodies. Moreover, the Bmax value of the binding of delta-receptor antagonist naltrindole to the delta-eGFP receptor (Scherrer et al., 2009; Scherrer et al., 2006) was found to be twice as high as the value in wild type mice, suggesting that the delta-EGFP receptor might therefore be different from the naïve one with respect to expression, subcellular localization and trafficking (Wang et al., 2008), further questioning the results obtained by Scherrer and colleagues (2009).

Taken together, further research needs to be done to solve the contradictory results of delta-receptor distribution. Electrophysiological recordings of DRG neurons after exposure of a specific delta agonist, for example, could be used to identify electrically and histochemically characterized A and C fiber types that respond to the agonist and, hence, express delta-receptors. However, the majority of data revealed that mu and delta-receptors are expressed in peptidergic C and Aδ fibers under naïve conditions and our results suggest that a nerve injury does not change the expression pattern of both receptors in the DRG and injured nerves.

Moreover, we have demonstrated the presence of the endogenous ligands of mu- and delta-receptors- β-endorphin and Met-enkephalin- in immune cells accumulating at the ligation site following nerve injury. The immune cells co-expressed CRF receptors (Labuz et al., 2009) which is know to cause opioid peptide release from leukocytes following CRF binding (Schafer et al., 1994). Importantly, we have recently shown that activation of CRF receptors by CRF application at the site of nerve injury results in full reversibility of the mechanical hypersensitivity evoked by nerve injury (Labuz et al., 2009). The CRF-induced analgesia was inhibited by prevention of immune cell accumulation using inter-cellular adhesion molecule (ICAM)-1 blockade. Reversibility of the analgesic effect was also achieved by injecting selective antagonists of all opioid receptor types near the nerve injury site or by a peripherally acting opioid receptor antagonist. The analgesic effect was thus

mediated by immune cell-released opioids activating opioid receptors expressed on primary sensory neurons at the ligation site. Our findings strongly point to a beneficial effect of inflammation associated with nerve damage through immune cell-derived endogenous opioids.

5.1.2. Expression of mu- and delta-receptors in the DRGs is not changed after nerve injury

We quantitatively investigated opioid receptor expression in the DRG, in the axons and the peripheral terminals of sensory afferent neurons, as these are the most relevant anatomical sites regarding the pain-relieving effects of peripheral opioid receptors. So far, only few studies assessed the expression of mu- and delta-receptor protein in the DRGs. In naïve animals, mu-receptor protein was found to be expressed in 18 – 20% (Ji et al., 1995; Kohno et al., 2005; Mousa et al., 2007) of rat DRG neurons, which is about half the number we found. However, in naïve mouse DRG neurons, around 39 – 45 % mu-receptor immunoreactive cells were found (Rashid et al., 2004; Truong et al., 2003; Yamamoto et al., 2008), which is consistent with the result in our study. The difference in the number of positive mu-receptor DRG neurons between rats and mice could be related to interspecies differences, which were already suggested regarding the expression pattern of opioid receptors (Mennicken et al., 2003). Delta-receptors were found in approximately 40–50% of naïve rat DRG neurons (Gendron et al., 2006), in contrast to two other studies reporting 13% or 15 % of delta-receptor immunoreactive neurons in naïve rat or mice DRG (Ji et al., 1995; Kabli and Cahill, 2007), which is consistent with our data.

Our quantitative analysis of the number of mu- or delta-receptor expressing DRG cells revealed no change at 2 days and 2 weeks following a CCI. Interestingly, studies using a nerve transection or the SNL and PSL nerve injury models reported either a down-regulation or, less often, an unchanged expression of mu-and delta-receptor protein in the spinal cord or DRGs (Besse et al., 1992; deGroot et al., 1997; Goff et al., 1998; Kohno et al., 2005; Porreca et al., 1998; Rashid et al., 2004; Stone et al., 2004; Zhang et al., 1998a; Zhang et al., 1998b), whereas, after a CCI, mu-receptor expression was shown to be rather upregulated (Besse et al., 1992; Kolesnikov et al., 2007; Stevens et al., 1991; Truong et al., 2003) and delta-receptors were found to be either decreased (Robertson et al., 1999; Stevens et al., 1991; Stone et al., 2004) or increased (Kabli and Cahill, 2007).

However, most of the abovementioned studies investigated the intensity of the expression of both receptors in the spinal cord. Very few studies examined expressional

changes of mu-and delta-receptors in the DRG after nerve injury, quantifying their mRNA or protein content. After a PSL, mu-opioid receptor mRNA was shown to be downregulated in mice DRG, whereas no change was found for delta mRNA (Pol et al., 2006). Two days up to 4 weeks after a CCI in rats, the mRNA content in the DRGs was down-regulated for all three receptors (Obara et al., 2009), was decreased for delta-receptors, but not changed for mu- and kappa-receptors (Herradon et al., 2008), or was similar for mu-receptors between the ipsilateral and contralateral DRGs (Truong et al., 2003). In summary, no clear picture develops concerning changes in the opioid receptor mRNA levels in the DRGs after nerve injury. However, regarding the functional relevance it is rather not the mRNA content but the protein expression level of the receptors that is of critical importance. Only two studies quantified the number of mu-receptor protein expressing neurons in the DRGs: one found an upregulation by 15-20% in the L5 DRG (Truong et al., 2003), the other one a very small increase within the L4 DRG that did not reach significance (Kolesnikov et al., 2007) at 2 and 14 days or 22 days after a CCI, respectively. Likewise, Kabli and Cahill identified a bilateral significant increase in the intensity of delta-receptor protein labelling in small and large L4-L6 DRG neurons 2 weeks after a CCI compared to sham-surgery, but no change in the number of delta-receptor expressing neurons (Kabli and Cahill, 2007). Considering all data, it seems that expressional changes of opioid receptors in the peripheral nervous system following nerve injury appear to be complex. They seem to be strongly dependent on the type and degree of the injury (tight ligation of SNL and PSL versus a loose ligation of the CCI) and the site and the parameter (e.g. staining intensity versus increase in positively-stained cell number) investigated. Albeit, no changes or up-regulation but no down-regulation of opioid receptor protein (regardless of the changes in the mRNA content) have been reported in the DRGs after a CCI. In line with this, our results suggest that the number of mu- and delta-receptor expressing cell bodies in the L4 and L5 DRGs of sensory fibers is not significantly changed 2 days and 2 weeks following a CCI in the sciatic nerve. However, we can not exclude an increase in the intensity of receptor expression, which seems to be highly probable considering the strong increase in the intensity of both receptors directly at the injured nerve.

5.1.3. Mu- and delta-receptor immunoreactivity is strongly up-regulated at the nerve injury site

Both, mu- and delta-receptor immunoreactivities were strongly enhanced proximally to the ligatures in the injured nerves at 2 days and 2 weeks following CCI, regardless of the lack of a higher number of mu- and delta-receptor expressing DRG cells. This suggests a

transport to and an accumulation of opioid receptors at the nerve injury site. A few studies, investigating the transport of receptors in naïve animals, reported a build-up of mu-receptors at a ligature induced in the vagus and sciatic nerve (McLean, 1988; Zarbin et al., 1990). Similarly, mu-and delta-receptor accumulation has been shown 2 hours after ligating the vagus or sciatic nerve, indicating stronger proximal densities (Ji et al., 1995; Li et al., 1996) or with a ratio of proximal to distal densities of about 5 to 1 (Young et al., 1980). The stronger proximal arrestment points to a predominantly anterograde axonal transport of opioid receptors, which is in line with our result. Interestingly, a potentiation of axonal transport of only mu or mu- and delta-receptors (measured as a more pronounced increase in β-endorphin- or DAMGO-binding sites 2 days after induction of a ligature) has been reported under inflammatory conditions (Hassan et al., 1993; Mousa et al., 2007). The latter study demonstrated an enhanced increase in anterogradely transported mu-receptors and provided evidence that this was mediated via an inflammation-induced retrograde transport of NGF from the inflamed paw tissue towards the DRG (Mousa et al., 2007). As a CCI involves an inflammatory response, including immune cell accumulation at the nerve injury site (see 5.1.1) a similar mechanism might be responsible for a CCI-induced enhancement of axonal opioid receptor transport in our experiments.

Opioid receptor expression at a nerve injury site has been, so far, hardly investigated. Western blot analysis of delta-receptor expression in the ipsilateral sciatic nerve after a CCI revealed an increase compared to the contralateral side and sham-operated animals (Kabli and Cahill, 2007), not stating the exact site of the increase. Using the same method, another study reported a mu-receptor protein accumulation proximal and distal to the ligature at 2 weeks after a CCI, with a stronger increase at the distal site (Truong et al., 2003). However, the use of Western Blot prevents any conclusion on the cell type expressing the opioid receptor and therefore from assessing specifically neuronal opioid receptors, which are relevant for mediating analgesic effects. The inclusion of opioid receptors on immune cells (Zollner and Stein, 2007) into analysis might have been one reason for a stronger distal increase of mu-receptors after a CCI (Truong et al., 2003) in opposite to stronger proximal increases in our and other abovementioned studies. In the present study, the increased neuronal immunoreactivity of mu-and delta-receptors proximal to a CCI is likely a result of injury-induced enhanced anterograde axonal transport of the receptor proteins, which get arrested at the first ligature. It is not clear why the increase of delta-receptor immunoreactivity at 2 weeks compared to 2 days following nerve injury is significantly lower. Under inflammatory conditions, the proximal accumulation of opioid receptors was highest at 12 hours and was

lowered 48 hours after induction of the ligature (Ji et al., 1995). It is therefore possible that the accumulation is also declining with time following nerve injury. However, the question remains, why this does not seem to affect mu-receptor immunoreactivities.

The number of fibers expressing mu-receptors proximal to the nerve ligature and in the ipsilateral paw tissue was not changed at 2 days and 2 weeks postsurgery, whereas the number of delta-receptor expressing fibers slightly increased proximal to the ligature. This could be a result of a higher dependence on the anterograde transport of delta-receptors compared to mu-receptor. It has already been suggested that delta-receptors are the main type of the three receptors undergoing axonal transport, as its very early accumulation at 2 or 12 hours after a ligation was found to be stronger than that of mu- and kappa-opioid receptors in naïve animals (Ji et al., 1995). While the expression of delta-receptors in same sensory fibers and their cell bodies might be normally under the detection limit of immunohistochemical staining, the arrestment and accumulation proximal to the nerve ligature could have increased the expression level of delta-receptors within these fibers above the detection limit.

5.1.4. Mu- and delta-receptor-expressing fibers in the hind paws

The number of mu-receptor expressing fibers in the paw was not changed, whereas the number of delta-receptor positive fibers partly decreased in the ipsilateral paw tissue. Recent studies using Western blot analysis reported an increase of mu-receptors in the hind paw skin innervated by the damaged saphenous nerve following CCI (Walczak et al., 2006). However, this study used Western blot for quantification and consequently, it is unclear whether opioid receptors were up-regulated in sensory nerve terminals. Moreover, the method did not allow assessing alterations in the number of mu-receptor expressing nerve fibers.

The Wallerian degeneration of sensory fibers distal to the nerve injury consequently leads to a lower number of fibers innervating the skin (Koeppen, 2004), which could have affected delta-receptor positive fibers. However, regarding the degree of degeneration in the CCI of the sciatic nerve a preferential degeneration of large myelinated fibers (Aβ) and a majority of small myelinated axons (Aδ) has been reported, whereas a variable but large percentage of the nerve's unmyelinated axons (C) remained intact (Basbaum et al., 1991; Bennett, 1993; Gautron et al., 1990; Munger et al., 1992). This suggests that the number of mu-and delta-receptors expressing C fibers are rather not affected on peripheral nerve terminals in the skin. Besides, one should consider the limits in sensitivity of the method used to validate small changes in the number of unmyelinated axons. C fibers are the only fiber type organised in Remark bundles, which comprise a certain number of C fibers surrounded

by Schwann cell processes (Murinson and Griffin, 2004). After a CCI, some Remark bundles were reported to contain a lower number of C fibers and in the most severely damaged nerve fascicles, C fibers were also found isolated and no longer clustered in Remark bundles (Basbaum et al., 1991). As C fibers approach the limit of resolution in the light microscope (Munger et al., 1992), it is likely that we would have not been able to quantitatively assess a loss in the individual opioid receptor-positive fiber number per Remark bundle.

On the other hand, it is possible that delta-receptors are to a larger percentage expressed in Aδ fibers than mu-receptors, as more Aδ than C fibers degenerate after a CCI. This would serve as an explanation for a slight decrease of only delta-and not mu-receptor expressing sensory fibers in the skin. However, detailed histochemical information on the percentages of mu-and delta-receptors in Aδ fibers remain to be examined. Interestingly, studies using whole cell patch clamp recordings in spinal dorsal horn neurons from rats showed that the mu agonist DAMGO more effectively inhibited C fiber-induced excitatory postsynaptic currents (EPSCs) than Aδ-induced EPSCs, whereas C- and Aδ-EPSCs were equally inhibited by the delta agonist DPDPE (Ikoma et al., 2007). This could indicate a higher number of delta-receptor expressing Aδ fibers, as opioid receptors are largely localized to presynaptic terminals in the spinal cord (Abbadie et al., 2002; Gouarderes et al., 1991).

5.1.5. Relevance of changes in opioid receptor expression on sensory neurons regarding peripheral opioid analgesia

Selective activation of opioid receptors on peripheral nerves has a potential for effective analgesia devoid of centrally mediated side effects (Stein et al., 2003). So far, the effects of opioids that act on their peripheral opioid receptors under neuropathic pain conditions have been mainly investigated in animal models. A few reports observed no analgesic effects through activation of peripheral opioid receptors, injecting mu-receptor agonists intraplantarly (i.pl.) into the nerve-injured paw (Aley and Levine, 2002; Rashid et al., 2004; Whiteside et al., 2004). Interestingly, in two of these studies the dosis of the agonist was either not mentioned or very low and all three employed the PSL model. A lack in the analgesic efficacy of opioids in neuropathic pain was therefore attributed to a decrease of peripherally expressed opioid receptors that was reported for mu-and delta-receptor protein in the spinal nerve terminals or DRGs following a PSL, SNL or a transsection of the sciatic nerve (deGroot et al., 1997; Goff et al., 1998; Kohno et al., 2005; Porreca et al., 1998; Rashid et al., 2004; Stone et al., 2004; Zhang et al., 1998b)

However, alterations in the opioid receptor protein levels in the spinal cord might affect spinal or supraspinal analgesic efficacy, but play no major role in peripheral opioid receptor-mediated analgesia. Changes in the DRG opioid receptor content might be indicative but are also not always predictive. A strongly reduced number of mu-receptors in DRGs (Rashid et al., 2004) correlated with the lack of antinociceptive effects of i.pl. injected mu-receptor agonists following PSL (see above), however i.pl. injections into the paw or systemic injections of a peripherally acting mu-receptor agonist produced antinociceptive effects following a SNL (Guan et al., 2008; Pertovaara and Wei, 2001) regardless of a minor downregulation of mu-receptors in DRG neurons in the same model (Kohno et al., 2005). Whereas an increase in the number of mu-receptor expressing DRG neurons was reported (Truong et al., 2003), we and two other studies found no change in the number of mu- and/or delta-receptor expressing DRG neurons following a CCI (Kolesnikov et al., 2007, Kabli and Cahill, 2007). However, the vast majority of behavioural studies adressing the effects of mu- and delta-receptor activation in the PNS revealed effective reversal of hypersensitivity following a CCI (Kabli and Cahill, 2007; Martinez et al., 2002; Obara et al., 2009; Obara et al., 2004; Truong et al., 2003). Interestingly, Kabli and Cahill identified an increase in the intensity of delta-receptor protein labelling in small and large L4-L6 DRG neurons 2 weeks after a CCI. This might indicate that changes in the intensity of opioid receptors in the DRGs following nerve injury allow for a better conclusion on the analgesic efficacy. Even more likely, opioid receptors on peripheral axons and their terminals are of highest relevance to peripheral opioid antinccioeption and their alterations might be therefore better indicators for an efficient peripheral opioid analgesia. In line with this, an increase in the intensity of mu-receptors distally to the CCI ligature was associated with antinociception after morphine and DAMGO injected at the injury site (Truong et al., 2003). Likewise, an increase in delta-receptor intensity at the CCI site correlated with an analgesic effect of delta-receptor agonist application, however, the agonist was injected into the nerve-injured paw (Kabli and Cahill, 2007). Using Western blot analysis, both studies could not differentiate between the cell types expressing the activated opioid receptors. We could demonstrate that mu-and delta-receptors accumulate in nerve fibers at the ligation site following a CCI of the sciatic nerve. Moreover, we suggest that their local activation promises effective pain relieve, since the majority of these opioid receptors was found in nociceptive neurons.

Quantification of the number of mu-and delta-receptor expressing nociceptive fibers near a nerve injury site has yet not been calculated in previous studies. The differential results for mu-and delta receptors (no change compared to a small increase in the fiber number,

respectively) in the present study might indicate differential regulatory mechanisms for both receptors (discussed under 5.1.3), or that measurements of the immunoreactive intensities of both receptors are more relevant. Moreover, the intensity can be measured directly proximal to the ligature site, whereas this seems to be hardly possible for the fiber number. Similarly, we demonstrated that the number of mu-receptor expressing nerve fibers in the paw tissue was not changed, whereas delta-receptor expressing fibers were partly decreased. However, i.pl. injections of a delta-receptor agonist into the nerve-injured paw were demonstrated to elicit analgesic effects following CCI, whereas the agonist did not produce an analgesic effect in the nociceptive behaviour of naïve animals to noxious heat stimulation (Kabli and Cahill, 2007). This indicates that i.pl. injections of delta-receptor agonists reveal an analgesic efficacy following nerve injury that is stronger than under normal conditions. The reduction of delta-receptor expressing nerve fibers in the paw tissue found in this study rather suggests that the number of opioid receptor expressing fibers is not a good indicator for the analgesic efficiency of agonists. Again, we can not exclude an increase in the immunoreactive intensity of delta-receptors due to higher numbers per nerve fiber. Thus, more studies investigating changes in the immunoreactivity and, moreover, ligand accessibility and affinity to the receptors, as well as signalling of opioid receptors on their peripheral terminals are needed.

Taken together, sufficient evidence from behavioural studies support the analgesic efficacy of opioid-opioid receptor interactions in the periphery as medications for neuropathic pain. The analgesic efficacy might depend on the injury model used (CCI and SNL versus PSL), however our results suggest that a strong increase in opioid receptor immunoreactivity in nociceptive fibers from injured nerves might be one important factor for the attenuation of hypersensitivity following a nerve injury.

5.2. Mechanical sensitivity of primary sensory neurons before and after mu-opioid receptor activation following nerve injury

In the first part of our studies (discussed under 5.1) we provided evidence for the expression of opioid receptors in peptidergic, small- and medium-diameter primary sensory neurons and investigated expressional changes of both receptors in the DRG cell bodies, in the axons at the injury site and at the peripheral axonal terminals following a CCI. In this part, our goal was to elucidate the functional consequence of opioid receptor activation in nerve-injured peripheral neurons. For this purpose, we first investigated the impact of the nerve damage on sensory fiber responses to mechanical stimulation of the fiber's receptive fields, using the in vitro skin-nerve preparation. We then assessed the effects of activation of mu-receptors on the same sensory fiber responses through application of the mu-receptor agonist DAMGO to the receptive fields. Mu-receptors were chosen for investigations, as they are the main opioid receptor type implicated in opioid-mediated analgesia (Fields, 2004).

We attempted to perform electrophysiological recordings at 2 days and 2 weeks following CCI of the saphenous nerve. However, a recording of action potentials from sensory fibers following mechanical stimulation at 2 days after CCI was hardly possible. This is in line with the report by Walczak et al. (2006) and might be related to the extensive Wallerian degeneration of sensory fibers that was observed already during the first two days following nerve injury (Koeppen, 2004; Lin et al., 2001). An explanation for the presence of a behaviourally manifested mechanical hypersensitivity at the same time point (Bennett and Xie, 1988; Dowdall et al., 2005; Labuz et al., 2009; Walczak et al., 2006) could be the collateral sprouting of sensory fibers from intact neighbouring nerves into the injured region responding to the mechanical stimulation (Devor et al., 1979; Inbal et al., 1987). Although strong degeneration is still present at 2 weeks postinjury, a partial recovery of myelinated and nonmyelinated axons was observed at 12 to 15 days following CCI (Gabay and Tal, 2004; Jankowski et al., 2009; Ma and Bisby, 2000). This might have accounted for the better electrophysiological recording conditions observed in our experiments. All primary afferent neurons in the present study were therefore investigated at 2 weeks after nerve damage. However, as a full regeneration does not occur at this timepoint, they were most likely spared neurons. Our behavioural measurement verified that the induction of a CCI in the saphenous nerve of mice resulted in a profound mechanical hypersensitivity at 2 weeks following nerve injury, similar to the result of a previous report (Walczak et al., 2006).

5.2.1. A nerve injury slightly lowers the conduction velocity of myelinated fibers

Primary sensory afferents of uninjured and injured nerves were first classified based on their conduction velocity. The calculated mean conduction velocity for Aβ, Aδ and C fibers in the present study was in a similar range like in previous *in vitro* skin-nerve recordings from individual sensory fibers in mice (Koltzenburg et al., 1997; Milenkovic et al., 2008). After nerve injury, Aβ and Aδ fibers revealed a small decrease in their conduction velocity, whereas C fibers conducted at a similar speed. Investigating only unmyelinated fibers, a lowered conduction velocity for C fibers was found at 3-107 days after a CCI in vitro (Koltzenburg et al, 1994). In contrast, a decrease in the conduction velocity of only myelinated fibers has been measured 12-15 days after a CCI in vivo (Gabay and Tal, 2004), similar to our result. The lowered conduction velocity of myelinated fibers could be explained by an injury-induced loss in the myelin thickness, which is a parameter determining the conduction velocity of a fiber (Waxman, 1980).

5.2.2. A nerve injury does not affect thresholds or latencies to mechanical stimulation of peripheral sensory fibers

The baseline mechanical thresholds of Aβ (RAM and SAM), Aδ (AM nociceptors and D-hairs) and C fibers measured in our study are comparable to those in previous studies using skin-saphenous nerve preparations in mice (Koltzenburg et al., 1997; Milenkovic et al., 2008). Two weeks after the CCI operation we did not measure changes in the mechanical thresholds of any fiber type, including nociceptive A and C fibers. Few studies have examined the effect of a peripheral nerve injury on the mechanical threshold of primary sensory afferents. A reduction in the mechanical thresholds of C and Aδ fibers has been demonstrated 2 to 5 weeks after SNL by in vivo recordings of the rat's sural and plantar nerves (Shim et al., 2005). Also, lower thresholds of Aδ nociceptors were reported at 4-6 weeks after a transection of the saphenous nerve in *ex vivo* intracellular DRG recordings in mice. Concurrently however, no changes in the mechanical thresholds of C polymodal nociceptors were reported in the same study (Jankowski et al., 2009). In contrast, a CCI of the sciatic nerve in rats produced an elevation of the mechanical threshold of C fibers measured in the in vitro skin-nerve preparation (Koltzenburg et al., 1994). Collectively, although differences over time and/or between painful conditions might occur, no clear conclusion can be drawn from previous data on mechanical thresholds of nociceptors following nerve injury. Following inflammation, two studies reported lowered thresholds to mechanical stimulation of nociceptors (Randich et al.,

1997; Wenk et al., 2006), however most studies did not observe decreased mechanical thresholds during inflammatory conditions, after incision or in response to noxious mechanical stimulation in nociceptors innervating the skin of rats and mice (Banik and Brennan, 2004, 2008; Du et al., 2001; Handwerker et al., 1987a; Kirchhoff et al., 1990; Reeh et al., 1987; Schlegel et al., 2004). Our results suggest that primary sensory fibers are not sensitized with respect to mechanical thresholds in neuropathy, similar to the majority of results in inflammation. We were also interested whether the latency of sensory afferents might be a parameter influenced by peripheral nerve injury. The baseline values we obtained are in a similar range to those of a previous study (Milenkovic et al., 2008). The results of the present study provide evidence that a nerve injury does not influence the latency time of response to mechanical stimulation of primary sensory neurons.

5.2.3. A nerve injury resulted in lowered discharge rates of slowly-adapting A fibers

Interestingly, we could show that the discharge rate of slowly-adapting A and C fibers to increasing strength of mechanical stimulation was decreased or tended to decrease in the injured nerve compared to controls. No effect on the discharge rates of rapidly-adapting fiber types (RA and D-hairs) could be observed. The lowered discharge rate affected mainly the higher displacements of SA and AM fibers, however this was probably not related to the repetitive stimulation per se because we did not observe such changes in RAM and D-hair fibers. In the present study we chose an interstimulus period of 30 sec between the different displacement steps. Using repetitive mechanical stimulation of the same strength and an interstimulus period of 60 sec resulted in a constant action potential number of SA, AM and C fibers from naïve mice (Shin et al., 2003), proving that the fibers did not attenuate due to the stimulation. Also, other studies using similar experimental protocols found either no changes in the discharge rate of SAM, AM and C fibers in a mouse saphenous nerve (Milenkovic et al., 2008) or an increase in the C mechanonociceptor firing rate in a rat tibial nerve (Randich et al., 1997) following inflammation. Under neuropathic conditions, the discharge rate of Aδ and C fibers to a single suprathreshold mechanical stimulus was increased 2 to 5 weeks after a SNL in rats (Shim et al., 2005). In contrast, the discharge rate of SA fibers was reduced at stronger mechanical stimulation after axotomy and regeneration of the mouse's saphenous nerve (Jankowski et al., 2009), when a stimulation protocol of increasing mechanical forces similar to ours was used. Our results indicate that mechanical hypersensitivity in neuropathy is not manifested in the discharge rates of primary afferent neurons, including nociceptors.

5.2.4. DAMGO acting at peripheral opioid receptors increased mechanical thresholds and decreased discharges of cutaneous nociceptors following nerve injury

In the present study, we could show that DAMGO application onto the fibers' receptive field significantly increased the mechanical threshold to von Frey hair stimulation in a noticeable group of Aδ (AM fibers) and C fibers in injured nerves. Only very few Aδ and C fibers were responsive to DAMGO in non-operated and sham-operated nerves. This is in line with in vitro skin-tibial nerve recordings of C fibers from another study, showing reduced firing rates following peripherally applied morphine under inflammatory conditions, whereas no similar changes were seen in C fibers innervating untreated skin (Wenk et al., 2006). Moreover, morphine did reduce the spontaneous firing of C and Aδ fibers from ultraviolet-irritated skin, but not the firing from both fiber types in normal skin when stimulated to discharge by application of KCL solution to the receptive field (Andreev et al., 1994). Although a better efficiency of peripherally i.pl. injected opioids under neuropathic pain compared to uninjured conditions is also supported by behavioural studies (Kabli and Cahill, 2007; Pertovaara and Wei, 2001; Walker et al., 1999), the underlying mechanism is still unclear and awaits further research. A higher efficiency of DAMGO on receptive fields of fibers from injured nerves could be related to a disruption in the blood-nerve barrier as shown at the nerve injury site following SNL (Abram et al., 2006) or to a degeneration of Schwann cells observed along the nerve distally to the injury (Koeppen, 2004). As Schwann cell processes cover the free nerve endings of Aδ and C nociceptors, a reduction of these processes could have provided improved accessibility of local opioid receptors in the paw tissue. However, both phenomena have not yet been investigated in the hind paws. Moreover, recent studies using Western blot reported an up-regulation of mu-receptors in the hind paw skin innervated by the damaged saphenous nerve following CCI (Walczak et al., 2006). As Western blot does not allow the identification of cell types, it is unclear whether opioid receptors were up-regulated in nerve terminals and whether this might account for an enhanced efficacy of DAMGO we observed in injured nerves. We, in contrast, did not find an upregulation in the number of mu-receptor-expressing nerve fibers on the peripheral terminals of the injured sciatic nerve. However, this does not allow a conclusion on possible changes in the intensity of mu-receptors in the paw tissue.

Although the dose of DAMGO employed in this study was relatively high (100 μM), the mechanical thresholds of Aδ and C fibers returned to the basal levels following washout, suggesting that DAMGO did not produce adverse effects. Importantly, the increase in the thresholds of Aδ and C fibers was fully reversed by pre-application of CTOP, confirming

specific actions through mu-receptors. Furthermore, DAMGO decreased the discharge rates of C fibers in which it enhanced the thresholds. This points to one group of C fibers that expresses mu-receptors on their cellular surfaces and is therefore sensitive to application of the agonist DAMGO. The discharges of DAMGO-responsive AM nociceptors were also attenuated, albeit the effect was not statistically significant. This might be related to a relatively high response variability and a low percentage of AM nociceptors which responded to DAMGO with elevated von Frey thresholds. Alternatively, this may imply that the effects of DAMGO on the thresholds are not predictive for its effects on the discharge rate of AM nociceptors. A better DAMGO-mediated efficiency in suppressing C fiber compared to Aδ fiber firing is in line with a previous study on hind paw inflammation, reporting Morphine-induced attenuation of the discharge rate to mechanical stimulation only in C fibers (Wenk et al., 2006). The requirement of DAMGO to enhance mechanical thresholds and to decrease discharges of nociceptors in the present study was further supported by a lack of such effects after application of a control buffer in DAMGO-responsive C and AM fibers.

The threshold and the discharge rate to mechanical stimulation of Aβ fibers and rapidly adapting D-hairs were not altered by DAMGO. This result strongly correlates with our immunohistochemical detection of mu-receptors predominantly in peptidergic C and Aδ fibers after nerve injury and implies that mu-receptors expressed on these nociceptive A and C fibers are targeted in the opioid treatment of neuropathic pain. Previous evidence suggested already that opioid receptors are indeed expressed on sensory nerve fibers that signal pain (Rau et al., 2005; Silbert et al., 2003). Moreover, although no other study directly tested opioid effects on cutaneous Aβ fiber responses, morphine applied on the spinal cord attenuated C fiber-, but not Aβ fiber-evoked dorsal horn neuron responses (Suzuki et al., 1999). Taken together, our results suggest that decreases in the discharge rates and increases in the threshold to mechanical stimulation of primary afferent nociceptors might represent a cellular mechanism of the reversal of mechanical hypersensitivity by peripherally applied opioids.

5.2.5. Relevance of mechanical sensitivity of cutaneous nociceptors and activation of their opioid receptors regarding peripheral opioid analgesia

In the present study, we first analyzed whether the behavioural hypersensitivity to mechanical stimuli after nerve injury is manifested by alterations in the primary afferent's threshold, the latency or the discharge rate to mechanical stimulation. Our results suggest that a peripheral nerve injury does not establish a hypersensitive state of residual skin-innervating

nociceptors with regard to their thresholds, latencies or discharge rates to mechanical stimulation. Interestingly, a sensitization to heat (in form of lowered heat thresholds and/or increased suprathreshold discharges to heat stimulation) has been detected in nociceptive C and/or Aδ fibers in a high number of studies exploring the effects of inflammation, noxious heat stimuli and burns in *in vivo* and *in vitro* recordings of trigeminal ganglions, sural, ulnar and saphenous nerves in monkeys, rabbits and rats (Beitel and Dubner, 1976; Fitzgerald, 1979; Kirchhoff et al., 1990; Kocher et al., 1987; LaMotte et al., 1982; Meyer and Campbell, 1981). Hence, the lack of a reduction in the thresholds or increase in the discharge rate following mechanical stimulation might be surprising, especially as mechanical sensitization is by far the most prominent feature of behaviourally manifested cutaneous hypersensitivity (Woolf and Mannion, 1999). One possibility is that sensitization to mechanical stimuli in form of reduced mechanical thresholds appears only in a small subset of nociceptors and doesn't affect the threshold of the whole population. The mechanical thresholds of TRPA1-positive C fibers, for example, were significantly lowered in inflamed skin, whereas there was no difference in the threshold of TRPA1-negative and in the combined group of C fibers (Dunham et al., 2008). Furthermore, we can not exclude a possible sensitization of uninjured nociceptors from neighbouring nerves that could be detectable with these parameters. C fibers from neighbouring, uninjured nerves have already been shown to develop e.g. spontaneous activity in response to nearby injury (Ali et al., 1999; Wu et al., 2001). Moreover, mechanical hypersensitivity of primary afferent nociceptors could be manifested in other parameters. One of these might involve the development of mechanosensitivity of so-called sleeping, previously mechanically insensitive afferents, which was shown following inflammation (Schmelz et al., 1994; Schmidt et al., 1995). This newly acquired mechanosensitivity of some sensory fibers would lead to an increased spatial summation of nociceptive input into the spinal cord. Our experiments do not allow any conclusion on alterations of these fibers, as mechanical probing with a glass rod prevented the inclusion of mechanically insensitive afferents in our population studied.

However, it is also very likely that not peripheral but central sensitization is mainly responsible for post-injury hypersensitivity of peripheral tissue (Woolf, 1983). The central terminals of low-threshold Aβ fibers, for example, were shown to sprout into lamina II of the spinal dorsal horn where nociceptive C-fibers normally terminate (Woolf et al., 1992), thereby mediating pain in response to normally innocuous stimuli. Recent investigations have provided strong evidence that spinal dorsal horn alterations in another neuronal population, the low-threshold C mechanoreceptors, could be responsible for a painful experience of low-

threshold fiber-activating stimuli after inflammation or nerve injury (Seal et al., 2009). However, as both fiber types were not shown to be sensitive to DAMGO in our study, their injury-induced spinal alterations consequently provide no explanation for an opioid-mediated analgesia. Another phenomenon thought to drive central sensitization after nerve injury is spontaneous activity, which has been observed in injured A and C fibers and uninjured C fibers after CCI, SNL and spinal nerve axotomy (Djouhri et al., 2006; Kajander et al., 1992; Shim et al., 2005; Wu et al., 2001). Early spontaneous activity of primary sensory C and A fibers, including nociceptors, was proposed as a key mechanism triggering the development of neuropathic pain behaviour following SNL or CCI (Xie et al., 2005). As we focussed on mechanically-induced responses, we did not investigate spontaneous activity in the present study. However, we could recognize ongoing activity after a CCI in a small number of fibers from injured nerves. As we hardly could detect a respective mechanically sensitive receptive field in the skin, it is likely that the activity originated at the nerve injury site. This would be in accordance to *in vivo* extracellular measurements from dorsal roots, which have provided evidence that spontaneous activity originates mainly at the level of cell bodies and at the nerve injury site (Kajander et al., 1992; Wall and Devor, 1983). In addition to ectopic activity, other mechanism including decreased inhibitory GABA levels in the dorsal horn following a CCI (Moore et al., 2002) or injury-induced increased releases of transmitters like SP and CGRP (Jang et al., 2004) from primary nociceptive neurons at the spinal cord level lead to a hypersensitization of secondary dorsal horn neurons. This results in exacerbated spinal responses to noxious stimuli (Basbaum et al., 2009) and elevated supraspinal pain perception. One way to overcome the elevated pain perception is a reduction of the nociceptive input. Our results suggest that DAMGO, following nerve injury, decreases the input of primary afferent nociceptors to the spinal cord by increasing their mechanical thresholds and lowering their discharge rates to mechanical stimulation. Thus, the inhibition of action potential generation or propagation in nociceptors might constitute a cellular mechanism of peripheral opioid receptor-mediated alleviation of mechanical hypersensitivity following nerve injury. As we found an unaltered number of mu-receptor expressing sensory fibers in the paw tissue, other, yet unknown mechanisms are likely responsible for the increased efficacy of DAMGO administration onto the receptive fields of fibers from injured compared to uninjured nerves. These could include an increase in the amount of mu-receptors per fiber, as we observed at the ligation side, a better accessibility of mu-receptors, as well as enhanced agonist affinity and receptor signalling.

5.3. Future studies

Taken together, our results indicate that following a CCI, the majority of mu-and delta-receptors remain expressed in peptidergic C and Aδ fibers which comprise predominantly fibers signalling nociceptive input. Moreover, both receptors accumulate proximal to the injury site after a CCI of the sciatic nerve, whereas the number of receptor-expressing fibers stays constant in the DRGs and within the paw tissue (except for a decrease in delta-expressing fibers). The increase in mu- and delta-receptor immunoreactivity proximal to the CCI could be based on an injury-induced enhanced antereograde transport and an arrestment of receptor protein at the ligature. With respect to their functionality, behavioural tests revealed that the mu-receptor agonist DAMGO and morphine enhanced von Frey thresholds after a CCI when injected in low (systemically inactive) doses directly at the site of nerve injury in rats (Truong et al., 2003). Moreover, preliminary behavioural data obtained in our group suggest that near-nerve injections of opioid receptor agonists are even more efficient in reversing mechanical hypersensitivity in mice compared to injections into the hind paw (D.Labuz, unpublished data). More studies investigating alterations of opioid receptor immunoreactivity including studies on their accessibility, on opioid – opioid receptor coupling and opioid receptor signalling along the injured axons and on their peripheral terminals could shed more light on possible mechanisms underlying this observation. For this purpose, it could be interesting to investigate electrophysiological recordings from C and Aδ fiber responses to mechanical stimulation after application of opioid agonists directly to the nerve injury site versus application to the receptive fields. The higher intensity of mu-receptors at the nerve injury site without an increase in the number of mu-receptor expressing fibers indicates that the amount of receptors per fiber is increased proximal to the injury. An elevation in opioid-induced nociceptive fiber responses (e.g. a stronger increase in the threshold and/or a stronger decrease in the discharge rate) following near nerve compared to receptive field application would provide evidence for an expression at the axonal cell membrane and an unhampered accessibility of the opioid receptors.

Inducing a CCI on the saphenous nerve did not result in a hypersensitive state of nociceptive (and non-nociceptive) fibers regarding their thresholds, latencies and discharge rates to mechanical stimulation. This suggests that the behaviourally observed mechanical hypersensitivity is not manifested in the mechanical-induced responses of nociceptors from injured nerves. However, the interesting question remains, whether nociceptive fibers from uninjured, neighbouring nerves establish a mechanical hypersensitivity in response to nearby injury that might be manifested in these parameters. Neighbouring nerves seem to be highly

impacted by the environmental changes following nerve injuries. Fibers from intact nerves are known to develop ectopic activity (Ali et al., 1999; Wu et al., 2001) and to increase their DRG mRNA content of neuropeptides in response to nearby injury (Fukuoka et al., 1998). Moreover, sensory fibers from intact neighbouring were found to sprout into the injured region (Devor et al., 1979; Inbal et al., 1987). However, there is no information on the impact of uninjured, neighbouring fibers on the behaviourally observed mechanical hypersensitivity following a nerve injury. Making use of the *in vitro* skin-nerve preparation, future experiments could investigate this question by recording mechanically-induced responses from singly sensory fibers of healthy nerves following injury of neighbouring nerves. Furthermore, evaluation of the effects of opioids applied to the receptive fields of nociceptors from intact neighbouring nerves that sprouted into the innervation territory of injured nerves would be of great interest. This could identify possible other mechanisms underlying the analgesic effects mediated by peripheral opioid- opioid receptor interactions.

6 Summary/ Zusammenfassung

Summary

Neuropathic pain arises as a direct consequence of a lesion or disease affecting the somatosensory system. Such pain can be spontaneous, occurring without external stimulation or stimulus-evoked. The latter can be manifested in a behavioural hypersensitivity to mechanical stimulation, which is frequently observed in patients or in animal models of neuropathic pain. Currently used centrally acting opioids are often associated with CNS-mediated side effects, which can be avoided by selective activation of opioid receptors on peripheral nerves. Therefore, the major goal of the present study was to investigate the expression pattern of peripheral opioid receptors and the effects of their activation on the thresholds, discharge rates and latencies to mechanical stimulation of primary sensory neurons following nerve injury. In a chronic constriction injury (CCI) of the sciatic nerve the expression of mu- and delta-receptors in the dorsal root ganglia (DRG), in the sciatic nerves and in the hind paws was examined with immunofluorescence. Additionally, the co-expression of opioid receptors with different sensory neuronal markers was evaluated by double immunofluorescence. To investigate the functional relevance of peripheral opioid receptor activation in neuropathic pain an *in vitro* skin-nerve preparation for single sensory fiber recording was used. Effects of mu-receptor agonist DAMGO on von Frey-induced mechanical thresholds and on nanomotor-induced mechanical stimulation to increasing forces were tested.

Whereas the number of DRG cells expressing mu- and delta-receptors did not change following CCI, the intensity of mu- and delta-receptor staining was greatly enhanced in the sciatic nerves proximal to the ligatures. The number of delta-receptor expressing fibers was slightly increased proximal to the nerve injury site and partly decreased in the paw tissue, in contrast to a consistent number of mu-receptor expressing fibers. Both opioid receptors were found in peptidergic Aδ and C fibers that mainly comprise nociceptors. Between 30- 45% of nociceptive Aδ and C fibers, but no Aβ fibers from injured nerves were responsive to DAMGO application onto their receptive field, demonstrating increased mechanical thresholds and a decreased number of action potentials in C fibers. The nerve injury itself did not change the thresholds and latencies of peripheral sensory fibers to mechanical stimulation, while their discharge rate was diminished. The results of the present study suggest that behaviorally manifested mechanical hypersensitivity does not require a sensitized state of

cutaneous nociceptors following nerve injury. Yet, enhanced thresholds and lowered discharge rates of nociceptors might constitute a cellular mechanism underlying peripheral opioid-mediated blockade of mechanical hypersensitivity in neuropathic pain. Increases in the amount of opioid receptors in nociceptive fibers at the ligature might indicate an enhancement of this effect by agonist injection near the injury site.

Zusammenfassung

Neuropathischer Schmerz resultiert aus einer Läsion oder einer Krankheit, die im somatosensorischen Nervensystem auftritt. Die Schmerzen können spontan, ohne externe Stimulation, oder durch eine erhöhte Reaktion auf bestimmte Stimulationsarten entstehen. Eine Sensibilisierung auf mechanische Stimulation wird sehr häufig in Patienten oder in Tiermodellen des neuropathischen Schmerzes beobachtet. Die Behandlung mit systemisch verabreichten Opioiden ruft häufig schwere Nebenwirkungen hervor, welche durch selektive Aktivierung von Opioidrezeptoren im peripheren Nervensystem verhindert werden können. Das Ziel dieser Studie war es daher, die Expression peripherer Opioidrezeptoren, sowie die Effekte ihrer Aktivierung auf die durch mechanische Stimulation hervorgerufenen Schwellenwerte, Anzahl der Aktionspotentiale und Latenzzeiten von primären afferenten Neuronen nach einer Nervenverletzung zu untersuchen. Nach Induzierung einer losen, chronischen Ligation (CCI) des Ischiasnervens wurde die Expression der Opioidrezeptoren mu und delta im dorsalen Wurzelganglion (DRG), im Ischiasnerv und in der Haut der Hinterpfoten mittels Immunfloreszenz untersucht. Des Weiteren wurde die Co-Expression der Opioidrezeptoren und verschiedener neuronaler Marker in Doppel-Immunfloreszenz-Experimenten ermittelt. Um die funktionelle Relevanz der Aktivierung peripherer Opioidrezeptoren zu untersuchen, wurde eine *in vitro* Haut-Nerv Präparation zur Messung einzelner sensorischer Neuronen genutzt. Getestet wurde die Wirkung des mu-Rezeptor Agonisten DAMGO auf die von Frey-Haar-induzierten mechanischen Schwellenwerte und auf eine Nanomotor-induzierte ansteigende mechanische Stimulation.

Die Anzahl der mu-und delta-Rezeptor exprimierenden DRG Neurone blieb nach der CCI Ligation konstant, wohingegen die Intensität beider Rezeptoren proximal zur Ligationsstelle stark anstieg. Die Anzahl der delta-Rezeptor exprimierenden Nervenfasern stieg proximal zur Verletzung an und nahm im Pfotengewebe leicht ab, stattdessen wurde eine gleichbleibende mu-Rezeptor exprimierende Faseranzahl an beiden Stellen festgestellt. Beide Opioidrezeptor-Typen wurden in peptidergen Aδ und C Nervenfasern gefunden, die hauptsächlich schmerzleitende Fasern (Nozizeptoren) beinhalten. Zwischen 30- 45% der

nozizeptiven Aδ und C Fasern, aber keine Aβ Faser der verletzten Nerven reagierten auf die Verabreichung von DAMGO auf ihr rezeptives Feld mit erhöhten mechanischen Schwellenwerten und einer Abnahme der Anzahl der Aktionspotentiale im Falle der C Fasern. Die Nervenverletzung selbst führte zu keiner Veränderung der mechanisch-induzierten Schwellenwerte und Latenzzeiten der Nervenfasern, während die Rate ihrer Aktionspotenziale abnahm. Die Ergebnisse der vorliegenden Studie weisen darauf hin, dass eine im Verhalten sichtbare mechanische Hypersensitivität keines sensibilisierten Zustandes kutaner Nozizeptoren bedarf. Erhöhte mechanische Schwellenwerte und eine Erniedrigung der Aktionspotenzialrate von Nozizeptoren könnten jedoch ein zellulärer Mechanismus sein, der der peripheren Opioid-vermittelten Hemmung mechanischer Hypersensitivität bei neuropathischen Schmerzen zugrunde liegt. Die Zunahme der Opioidrezeptoren in nozizeptiven Fasern an der Ligationsstelle deutet an, dass dieser Opioidrezeptor-vermittelte Effekt nach Injektion von Agonisten nahe der Nervenverletzung stärker ausfallen könnte.

7 References

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8   Publications and Conferences

Publications

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