Charles University

Faculty of Science

Study programme: Animal physiology

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The role of nociceptive synaptic transmission modulation

Úloha modulace nociceptivního synaptického přenosu

Doctoral thesis

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Prague 2022

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Acknowledgment

I would like to express my deepest gratitude to my supervisor MUDr. Jiří Paleček, CSc., for his invaluable patience and feedback. This endeavor would not have been possible without all of my co-workers from the Laboratory of Pain research, Institute of Physiology, CAS, namely RNDr. Pavel Adámek, Ph.D, Mgr. Diana Špicarová, Ph.D., RNDr. Petra Mrózková, Ph.D., and Kateřina Krämerová.

I am also thankful to my partner, Ing. Bibiána Juhászová, for her neverending patience and moral support. Thanks also go to my parents for their faith in me and their emotional support.

Abstrakt

Bolest je hlavním příznakem mnoha onemocnění a často se může stát hlavním negativním faktorem nízké kvality života pacienta. Komplexní problematiku léčby bolesti dále podtrhuje snížená účinnost konvenčních analgetik např. u neuropatické bolesti. Neuropatická bolest na rozdíl od akutní nociceptivní bolesti vzniká v důsledku poškození periferního nebo centrálního nervového systému a často přechází v syndrom chronické bolesti. Většina dnes dostupných analgetik poskytuje u chronické neuropatické bolesti pouze omezenou a neuspokojivou analgezii, která je často spojena se závažnými nežádoucími účinky.

Modulace nociceptivního přenosu v zadním rohu míchy (SCDH) je považována za klíčový mechanismus při vzniku a udržování zejména chronické bolesti. Hlavním cílem této disertační práce bylo studovat, jak modulační procesy synaptického přenosu v zadním rohu míšním ovlivňují analgezii navozenou opioidy. Práce byla zaměřena zejména na interakci mezi CCL2 (z angl. chemokine (C-C motif) ligand 2), TRPV1 (z angl. transient receptor potential vanilloid type 1) a μ-opioidním receptorem (MOR). Pro pochopení modulačních vlivů opioidní signalizace v SCDH jsme si položili následující otázky: (I.) Jak CCL2 moduluje účinky aktivace MOR na nociceptivní synaptický přenos v neuronech SCDH in vitro a in vivo během opioidy zprostředkované analgezie? (II.) Jsou účinky zprostředkované CCL2 závislé na aktivaci mikroglií? (III.) Jaká je role receptorů TRPV1 při vzniku opioidy indukované hyperalgezie (OIH) a jak může aktivace TRPV1 endogenním agonistou N-oleoyldopaminem (OLDA) modulovat desenzitizaci MOR? Druhým cílem práce bylo studovat úlohu fosfatidylinositol 3-kinázy (PI3K) v rozvoji neuropatické bolesti vyvolané chemoterapií paclitaxelem (PAC).

Jako primární metoda pro zkoumání modulace nociceptivního přenosu v SCDH byla použita metoda terčíkového zámku (patch-clamp) snímání excitačních postsynaptických proudů (EPSCs) z nociceptivních neuronů zadního rohu v akutních míšních řezech. Elektrofyziologická data byla doplněna behaviorálními experimenty a imunohistochemickou analýzou tkání.

Naše výsledky ukázaly, že aplikace MOR specifického agonisty [D-Ala2, N-MePhe4, Gly-ol]-enkefalin (DAMGO) vede k silné inhibici excitačního nociceptivního přenosu a snížení citlivosti na tepelné podněty (analgezii). Dále jsme ukázali, že analgezie

zprostředkovaná DAMGO je výrazně oslabena po aplikaci CCL2, a tento efekt je závislý na aktivaci mikroglií. Dále jsme ukázali nutnost aktivace receptoru TRPV1 pro rozvoj opioidy navozené hyperalgezie (OIH), v našich experimentech sledované jako opožděná potenciace evokovaných EPSC v neuronech SCDH. Naše dosud nepublikované výsledky z experimentů zaměřených na interakci TRPV1-MOR naznačují, že aktivace TRPV1 endogenním agonistou může snížit jak DAMGO, tak CCL2 indukovanou desenzitizaci MOR. Výsledky dvou studií zaměřených na periferní neuropatii vyvolanou PAC (PIPN) potvrzují významné zvýšení aktivity PI3K a známek neuroinflamace ve spinálních gangliích (DRG). Důležité je zejména zjištění, že lék schválený ke klinickému použití Duvelisib, specifický inhibitor PI3K, může snížením aktivity PI3K výrazně omezit infiltraci/proliferaci makrofágů v DRG.

Výsledky tohoto doktorského projektu odhalují nové potenciální cesty ve výzkumu léčby bolesti a zdůrazňují ústřední roli TRPV1, CCL2 a PI3K v modulaci nociceptivního přenosu. Další výzkum těchto základních mechanismů modulace nociceptivní signalizace by mohl přispět ke zlepšení analgetické léčby pacientů.

Klíčová slova: bolest, nocicepce, neuropatická bolest, CCL2, opioidy, TRPV1, paclitaxel, PI3K

Abstract

Pain represents a major symptom in a multitude of medical conditions and can often become the main negative factor in a patient's low quality of life. The complex issue of pain management is further underscored by the reduced efficacy of conventional analgesics in conditions such as neuropathic pain. Neuropathic pain, unlike acute nociceptive pain, originates from damage to the peripheral or central nervous system and often develops into chronic pain syndrome. Most analgesics available today provide only limited and unsatisfactory analgesia in chronic neuropathic pain and are often associated with severe adverse effects.

Modulation of nociceptive transmission in spinal cord dorsal horn (SCDH) stands out in recent research as a pivotal mechanism, especially in chronic pain development and maintenance. The major aim of this doctoral thesis was to investigate how pain-associated processes interfere with opioid-induced analgesia, with the main focus on the interaction between chemokine (C-C motif) ligand 2 (CCL2), transient receptor potential vanilloid type 1 (TRPV1), and μ-opioid receptor (MOR). To achieve a better insight into opioid signaling in SCDH we studied the following issues: (I.) How does CCL2 modulate MOR-mediated effects on nociceptive synaptic transmission in SCDH neurons and *in vivo* during opioid-mediated analgesia? (II.) Are CCL2-mediated effects dependent on microglia activation? (III.) What is the role of TRPV1 receptors in the development of opioid-induced hyperalgesia (OIH) and how can the activation of TRPV1 by endogenous agonist N-Oleoyldopamine (OLDA) modulate MOR desensitization? The second part of the thesis aimed to study the role of phosphatidylinositol 3-kinase (PI3K) in the development of neuropathic pain induced by paclitaxel (PAC) chemotherapy.

Whole-cell patch-clamp recordings of excitatory postsynaptic currents (EPSCs) recorded from superficial dorsal horn neurons in acute spinal cord slices were used as a primary method to investigate nociceptive transmission. Electrophysiological data were supplemented by behavioral experiments and immunohistochemistry.

Our results demonstrate that the application of MOR-specific agonist [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO) exhibits potent inhibitory control of excitatory nociceptive transmission and robust hyposensitivity to thermal stimuli (thermal analgesia). We showed that DAMGO-mediated analgesia is severely attenuated after the application of

CCL2 in a microglia-dependent manner. Furthermore, we showed that TRPV1 receptor activation is required for the OIH development, evident in our experiments as a delayed potentiation of electrically evoked EPSCs. Our unpublished data from experiments aimed at TRPV1-MOR interaction suggest that activation of TRPV1 can diminish both agonist- and CCL2-induced MOR desensitization. Results from two studies aimed at PAC-induced peripheral neuropathy (PIPN) confirm a significant PAC-induced increase in PI3K activity and neuroinflammatory factors in dorsal root ganglia (DRG). Most importantly, these results demonstrate that clinically approved PI3K inhibitor Duvelisib can attenuate PI3K activity as well as macrophage infiltration/proliferation in DRGs.

Taken together, the results of this Ph.D. project reveal new potential avenues in pain management research by highlighting the central role of TRPV1, CCL2, and PI3K in the nociceptive transmission modulation. Further research of the underlying mechanisms of nociceptive transmission modulation could contribute to improved opioid-mediated analgesia.

List of keywords: pain, nociception, neuropathic pain, CCL2, opioids, TRPV1, paclitaxel, PI3K

List of Abbreviations

20:4-NAPE	Arachidonoylphosphatidylethanolamine
AKAP	A-kinase anchoring protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CB_1	Cannabinoid receptor 1
CCI	Chronic constriction injury
CCL2	Chemokine (C-C motif) ligand 2
CCL7	Chemokine (C-C motif) ligand 7
CCL8	Chemokine (C-C motif) ligand 8
CCL12	Chemokine (C-C motif) ligand 12
CCL13	Chemokine (C-C motif) ligand 13
CCR2	C-C motif chemokine receptor 2
CCR4	C-C motif chemokine receptor 4
CD68	Cluster of differentiation 68
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CIPN	Chemotherapy-induced peripheral neuropathy
CNS	Central nervous system
CX3CL1	Fractalkine
DAMGO	[D-Ala^2, *N*-$MePhe^4$, Gly-ol]-enkephalin
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DOR	δ-opioid receptor
DRG	Dorsal root ganglia
DUV	Duvelisib
eEPSC	Electrically-evoked excitatory postsynaptic current
EPSC	Excitatory postsynaptic current

ERK1/2	Extracellular signal-regulated kinase 1/2
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
GDP	Guanosine diphosphate
GIRK	G protein-coupled inwardly rectifying potassium channel
GLYT2	Glycine transporter 2
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
IASP	International Association for the Study of Pain
i.p.	Intraperitoneal
i.i.	Intrathecal
IR	Immunoreactive
KOR	κ-opioid receptor
LPS	Lipopolysaccharide
MCP1	Monocyte chemoattractant protein 1
mEPSC	Miniature excitatory postsynaptic current
Mrgprd	Mas-related G protein-coupled receptor D
MOR	μ-opioid receptor
NADA	N-arachidonoyldopamine
NDS	Normal donkey serum
NGF	Nerve growth factor
NK1R	Neurokinin 1 receptor
NMDA	N-methyl-D-aspartate
NOP	Nociceptin opioid peptide receptor
OLDA	N-Oleoyldopamine
OIH	Opioid-induced hyperalgesia
ORL1	Opioid receptor like receptor 1
$P2X_3$	P2X purinoreceptor 3
PAC	Paclitaxel
pAkt	Phosphorylated Akt kinase
PAR2	Protease-activated receptors 2
Pax2	Paired box gene 2
PBS	Phosphate-buffer solution

PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PIPN	Paclitaxel-induced peripheral neuropathy
PKA	Protein kinase A
PKC	Protein kinase C
PLCβ	Phospholipase C-β
PNS	Peripheral nervous system
PWL	Paw withdrawal latency
PWT	Paw withdrawal threshold
RM	Repeated measures
ROI	Region of interest
RTX	Resiniferatoxin
SCDH	Spinal cord dorsal horn
SCI	Spinal cord injury
SEM	Standard error of mean
sEPSC	Spontaneous excitatory postsynaptic current
SNL	Spinal nerve ligation
SP	Substance P
STZ	Streptozocin
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TRPV1	Transient receptor potential vanilloid type 1
TTX	Tetrodotoxin
VGAT	Vesicular GABA/glycine transporter
VGCC	Voltage-gated calcium channel
VR1	Vanilloid receptor 1
WMN	Wortmannin
XCL1	Chemokine (C motif) ligand 1
XCL2	Chemokine (C motif) ligand 2

Content

Introduction

Pain is defined as an unpleasant perceptual and emotional experience caused by actual or threatened tissue damage and has an undeniable physiological significance in protecting the body from harm. Noxious stimuli evoke acute nociceptive pain, detected by peripheral sensory neurons called nociceptors. Pain perception is always subjective and depends on momentary circumstances and the well-being of an individual exposed to a noxious stimulus. Persistent nociceptive stimulation can lead to a maladaptive modulation of nociceptive pathways, causing chronic pain, that extends beyond the expected period of healing.

Chronic pain affects up to 30-40% of the population and is very difficult to manage. The management of chronic pain conditions is a significant burden for patients and a major socioeconomic problem. Current analgesic treatment approaches carry with them a range of undesirable side effects and unsatisfactory pain relief for patients suffering from acute and especially chronic pain. Damage or disease affecting peripheral or central nociceptive pathways can also lead to chronic pain of neuropathic origin. This pathological neuropathic pain lacks physiological significance, is harmful to patients, and involves a wide range of underlying mechanisms.

The complexity of mechanisms involved in neuropathic pain development greatly complicates pain management by conventional analgesics. This is also due to the fact that neuropathic pain usually does not originate from normal activation of peripheral nociceptors and thus the effectivity of standard analgesics is very limited. For example, neuropathic pain in chemotherapy-induced peripheral neuropathy (CIPN) combines peripheral neuropathy, modulation of dorsal root ganglia (DRG) neurons function, and dorsal horn neurons sensitization, leading to allodynia and hyperalgesia. The available treatment of CIPN-related pain is thus highly unsatisfactory.

Opioid therapy, while a staple of acute pain treatment, provides only a very limited pain relief in neuropathic pain management. In addition, opioids have to be prescribed with great caution, as their use is accompanied by serious side effects including breathing

depression, addiction, or side effects that severely complicate opioid regimen such as opioid-induced hyperalgesia and opioid tolerance.

Regardless of the significant progress in neuropathic pain research, remaining gaps in our understanding of different painful conditions compromise effective pain treatment management. For this reason, the major aim of this Ph.D. thesis was to investigate mechanisms associated with neuropathic pain development and treatment, with a focus on the modulation of opioid-induced analgesia. Furthermore, experiments included in the project concentrated on the role of transient receptor potential vanilloid type 1 (TRPV1) receptor and its involvement in opioid receptor regulation, specifically opioid-induced hyperalgesia and the desensitization of opioid receptors. The second part of the project aimed to investigate changes in DRG associated with paclitaxel-induced peripheral neuropathy (PIPN) and potential targets for PIPN-induced neuropathic pain treatment. The experimental work described was published in three original articles that are attached at the end of the thesis as an Appendix.

Part I.

Literature review

1. Definition of Pain

Pain is defined by The International Association for the Study of Pain (IASP) as “An unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage.” Pain is an irreplaceable part of the body’s defense system, as it motivates the individual to withdraw from the harmful situation and avoid similar noxious stimuli in the future. As pain is a symptom of a vast number of underlying conditions, it is the most common reason why patients seek medical attention in developed countries. It is important to remember that apart from the sensory component of pain, pain has also an affective, emotional component, and is therefore perceived subjectively. Treatment of pain, therefore, requires a careful and individual approach by the physician; the difficulty to measure pain objectively and ambiguity in perception and description of pain by the patients greatly contribute to frequent mismanagement of pain itself or its underlying conditions.

There are several features by which pain can be classified. Of course, a region of the body where pain manifests is the first and foremost feature patients describe, followed by an organ or a system causing the pain. Intensity is the next and most difficult feature of pain to classify; because pain is of a subjective nature, many health care workers underestimate its severity. To emphasize the importance of a patient’s self-assessment of pain intensity, Margo McCaffery defined pain as “Pain is whatever the experiencing person says it is, existing whenever he says it does”. Following this logic, the intensity of pain is clinical practice assessed on a scale of 0 to 10, while questionaries help with assessing the quality of pain.

The better-defined and more straightforward feature used to classify pain is the duration of pain and pattern of occurrence of pain. Acute pain, as the name suggests, occurs suddenly after a nociceptor stimulation that exceeded the nociceptive threshold. The quality of acute pain is most often described as sharp and serves as a warning signal for actual or potential imminent damage. The intensity of acute pain can vary from mildly unpleasant to severe as it can originate from anything from an insignificant skin injury to a traumatic injury or serious disease. Therefore, while in most cases acute pain stops right after the noxious stimulus causing pain is removed, it can last for months until the underlying condition is healed completely.

Chronic pain is pain that may persist long after the underlying condition was healed or been removed. Chronic pain usually lasts longer than three months and can be hard to accurately diagnose as the cause of chronic pain is often ambiguous or difficult to identify. Studies from the United States, Canada, and European countries reveal that approximately 20 – 30 % of the population suffer from some form of chronic pain (Breivik et al., 2006; Johannes et al., 2010; Schopflocher et al., 2011), with high prevalence among individuals younger than 60 years (O'Connor, 2009).

Nociceptive pain is defined by IASP as *"pain that arises from actual or threatened damage to non-neuronal tissue and is due to activation of nociceptors"*. In general, nociceptive pain is caused by tissue damage detected by nociceptors. Nociceptive pain has a clear protective physiological function and is most commonly experienced in the musculoskeletal system. Damage eliciting nociceptive pain can be of physical or chemical nature or can be triggered by hot or cold temperatures.

2. Nociception and pain

2.1. Nociception

Nociception describes a process of neural encoding of noxious stimuli and requires nociceptors and nociceptive pathways to transduce noxious stimuli to the central nervous system (CNS). The existence of distinctive peripheral nerve fibers, specialized for the transduction of painful stimuli was first proposed at the beginning of the 20th century (Sheringhton, 1900). In his work, Sherrington defined the term nociception, described the necessary neural structures of nociceptive pathways, and even created a link between nociceptive reflexes and perception of pain (Woodworth et al., 1904).

Nociceptors are primary sensory neurons, with characteristic thresholds and sensitivity that allow them to be activated by noxious stimuli. A noxious stimulus can be represented by intense pressure, heat, or irritant chemical, and its detection by nociceptors is the first step in pain sensation; however, activation of nociceptors in itself does not necessarily lead to pain perception. Nociceptors serve only to encode and transduce noxious stimuli to higher processing centers and pain perception happens only if the signal is not inhibited at these centers and reaches the brain cortex.

Because the nociceptive information has to travel from the site of acute damage throughout several neural structures to its potential final destination in cortical regions of the brain, the nociceptive pathways are comprised of a wide variety of neuronal cells and employ a vast repertoire of receptors and signaling molecules. The following chapters review the main parts of a properly functioning nociceptive system.

2.2. Nociceptors

Nociceptors, as well as other primary afferent sensory neurons, convert environmental stimuli into electrochemical signals. Unique receptor composition allows nociceptors to detect both physical (pressure or heat) and chemical (change in pH levels) stimuli and at the same time to distinguish and integrate complex environmental stimuli (Julius et al., 2001). This ability is granted mainly due to polymodal receptors, such as TRPV1 which can transduce signals evoked by heat surpassing its threshold as well as by chemical irritation (capsaicin, acidic pH).

Nociceptor morphology is similar to other sensory afferent neurons; nociceptors are pseudo-unipolar neurons with cell bodies situated in DRG or trigeminal ganglia in the case of nociceptors innervating head (Kandel, 2013). Cell bodies inside ganglia are clustered with surrounding glial cells, providing necessary metabolic support. Axon forms T-shaped bifurcations with both branches acting as a single axon. Individual branches of the axon are referred to as a distal process and proximal process. The long peripheral process - the distal process - extends to the periphery where the axon forms sensory ending in the innervated organ. Nociceptive endings form in the skin, to collect stimuli from the environment, in muscles and joints where nociception protects the body from tissue damage resulting from exceeding the natural range of motion and in viscera.

Peripheral nerve fibers are generally classed into three major groups based on the diameter, signal conduction velocity, and myelination of the axons. Nociceptive information is under physiological conditions transmitted by Aδ and C fibers. Both fiber types are polymodal; react to and transduce more than one type of stimulus. Aδ and C fibers convey localized mechanical or thermal stimuli to the dorsal horn of the spinal cord. The main differences between Aδ and C fibers are in their myelination and cell body size, resulting in different conduction velocities and firing patterns.

Aδ fibers are small-diameter (1 to 6 μm), myelinated primary afferent fibers with conduction velocity between 3 to 30 $m*s^{-1}$. Due to their fast conduction velocity, Aδ fibers facilitate the perception of stimuli that evoke fast, sharp pain, referred to as first pain. Their peripheral endings consist of free nerve endings sensitive to pressure or are equipped with thermoreceptors sensitive to heat or cold. Two main classes of Aδ fibers can be distinguished by their response to intense heat; Aδ nociceptors respond to a temperature above 53°C (type I) or are activated at a temperature above 43°C (type II) (Julius et al., 2001).

C fibers are unmyelinated small-diameter (0,2 to 1,5 μm) primary afferent neurons with a slow conduction velocity of approximately only 2 $m*s^{-1}$. C fibers form synapses with second-order projection neurons in the upper laminae of the spinal cord dorsal horn. Polymodal C fibers react to mechanical, thermal, or chemical stimuli and even respond to internal physiological changes such as hypoxia, hypoglycemia, or buildup of metabolic products in skeletal muscle (Craig, 2003). A minor subpopulation of C fibers is insensitive to mechanical stimuli but still responds to noxious heat or chemical stimuli (Julius et al., 2001). Because of the slower conduction velocity, C fibers respond to stimuli of stronger intensities, and mediate “second” pain; long-lasting, burning, or dull pain, more diffuse than sharp pain mediated by rapidly conducting Aδ fibers. (Brooks et al., 2005).

Characterization of nociceptors in DRG is based on expression profiles and membrane protein composition of nociceptive markers. The most prominent markers are ion channels, especially the voltage-gated calcium, sodium, and potassium channels, modulating the excitability of nociceptors (Binzen et al., 2006). Nociceptors can be further divided into two major subpopulations based on the membrane receptor composition, functional properties, and composition of their secretory vesicles – peptidergic and non-peptidergic nociceptors (Woolf et al., 2007).

The major trait of peptidergic neurons is the expression of calcitonin gene-related peptide (CGRP) and substance P (SP). Most of the peptidergic terminals form synapses in spinal cord lamina I and lamina II_o, transducing signals to neurokinin 1 receptor (NK1R)-positive second-order neurons (Snider et al., 1998; Wang et al., 2009). A prominent and abundant receptor that differentiates peptidergic neurons from non-peptidergic neurons is the tyrosine kinase receptor for a nerve growth factor (NGF) – TrkA. Non-peptidergic

neurons were first classified based on their ability to bind the IB4 isolectin, which allows for highly selective immunostaining (Snider et al., 1998). IB4-positive non-peptidergic neurons further express the P2X purinoreceptor 3 ($P2X_3$) purinergic receptor, c-Ret neurotrophin receptor and almost 75% of non-peptidergic DRG neurons express the Mas-related G protein-coupled receptor D (Mrgprd). Non-peptidergic neurons form synapsis mostly with neurons located in lamina II_i (Snider et al., 1998; Wang et al., 2009).

2.3. Nociceptive pathways

2.3.1. Nociceptive processing in the spinal cord

Somatosensory information from peripheral and visceral organs enters CNS through spinal cord dorsal horn grey matter. Proper transmission and modulation of the peripheral input rely on a high organization of the circuits forming a connection between the periphery and the CNS. The system of laminar organization of layers of grey matter was first described in the early 1950s by Bror Rexed, describing the cytoarchitecture of cat spinal cord, and has been since widely adopted to describe the anatomy and functional organization of dorsal horn grey matter (Rexed, 1952).

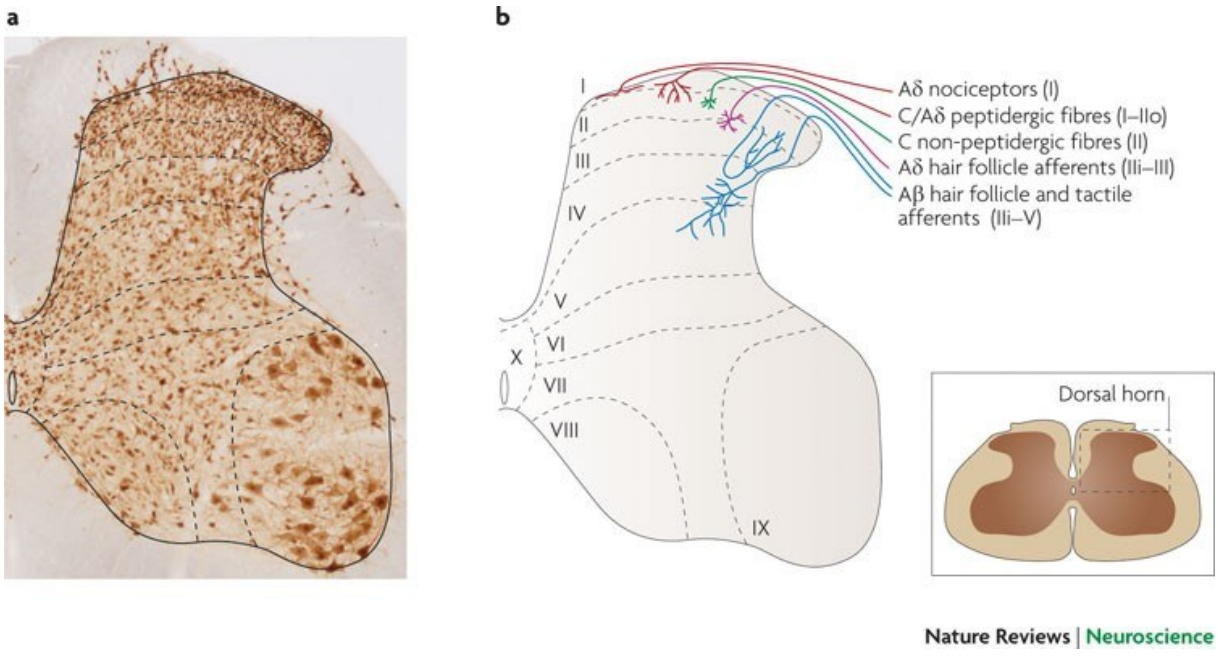

Figure 2.1.: **Representation of laminar organization of the dorsal horn with a schematic of afferent inputs.** (**a**) A representative image of a rat spinal cord slice. Immunostaining of neuronal soma using NeuN antibody shows a characteristic distribution of small neurons in superficial dorsal horn layers. Laminae's boundaries are

illustrated by dashed lines. (**b**) A schematic image of primary afferent fibers arborization in dorsal horn laminae. The laminar pattern illustrated depends on the fiber diameter and function. Adapted from (Todd, 2010).

Superficial laminae (laminae I and II) are the most important dorsal horn layers as nociceptive afferent fibers of Aδ and C-type form synapses with projection neurons in these laminae. Lamina I, also called the marginal zone, contains predominantly large-diameter projection neurons that receive input primarily from Aδ primary afferent fibers and C fibers. Lamina II, or substantia gelatinosa, was later further divided into lamina II_o or outer and lamina II_i or inner, based on observed differences in receptors expressed by the spinal neurons and distinct types of primary afferents ending in lamina II_o and lamina II_i. Most of the neurons identified in lamina II are spinal interneurons.

Although some of the nociceptive information from Aδ primary afferent fibers projects to lamina III and IV, these layers mostly receive the input from tactile fibers of type Aβ. Lamina III and IV also contain neuronal cell bodies and synapses of the spinothalamic tract. Nociceptive transmission from visceral organs, muscles, and ligaments is carried by afferent C fibers and enters CNS in lamina V. The last layer of the dorsal horn is lamina VI, referred to as nucleus dorsalis, and is responsible for the processing and transmission of fast pain-evoked reflexes. Intermediate grey matter in lamina VII and ventral horn formed by laminae VIII and IX, in general, do not receive nociceptive input, while the last layer, lamina X receives input from a small number of C and Aδ afferent fibers (Millan, 1999).

The proximal process forms a connection between peripheral endings by a synapse with a second-order neuron in the dorsal horn of the spinal cord or *subnucleus caudalis* in the brainstem (Dubin et al., 2010). Here is nociceptive information carried by a nociceptor first transmitted to the CNS and represents the first target of synaptic modulation of sensory information from peripheral and visceral organs. The synapse between the nociceptor and a projection neuron is a fast excitatory glutamatergic synapse, with postsynaptic *α*-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors activated by a presynaptic release of L-glutamate (Coggeshall et al., 1997; Erreger et al., 2004; Zeilhofer, 2005).

Projection neurons, neurons of the spinal cord responsible for the transmission of stimulus from afferent fibers to deeper laminae or the brain are predominantly located in

lamina I, V, and VI (McMahon et al., 2006). Projection neurons are of heterogenous nature, characterized by their morphology and characteristics of response to stimulation. The latter classification divides projection neurons into three groups based on the type of information they can receive and process. Low-threshold or non-nociceptive neurons process sensory input from Aβ low-threshold mechanoreceptors. The majority of low-threshold projection neurons are in laminae II-IV. High-threshold or nociceptive projection neurons receive a transmission from Aδ and C-fibers. High-threshold projection neurons are normally silent and can be activated only by nociceptors transmitting noxious stimulation. Nociceptive projection neurons can be found mainly in lamina I and II_o and to a lesser extent in laminae V and VI. The last class of projection neurons is referred to as wide-dynamic range neurons, due to their ability to receive and process direct inputs from Aβ and Aδ-fibers, but also polysynaptic input from C-fibers. Wide-dynamic range neurons are located in lamina V and converge peripheral and visceral nociceptive input (Basbaum et al., 2009).

The first modulation of nociceptive transmission is in form of neuromodulators released together with L-glutamate. Alteration in release vesicles composition can potentiate the strength of the synapse and modulate the excitability of postsynaptic neurons, as based on the type of the afferent neuron the vesicles can contain a wide range of pro-nociceptive neuromodulators such as CGRP, SP, neurokinin A (Khawaja et al., 1996; Sun et al., 2004), adenosine triphosphate (ATP) (Sawynok et al., 2003), various cytokines and interleukins (Spicarova et al., 2010; Kras et al., 2014; Spicarova et al., 2014; Zhu et al., 2014; Fang et al., 2015; Donnerer et al., 2018) growth factors (Bonnington et al., 2003) and other neuropeptides and metabolites of phospholipids.

Nociceptive synapse is further modulated by inhibitory spinal interneurons, forming synapses with dorsal horn projection neurons before the transmission to the reflex circuits and the brain (Gutierrez-Mecinas et al., 2018). Principal neurotransmitters of inhibitory interneurons are γ-aminobutyric acid (GABA) and glycine. Their population in rat dorsal horn lamina I, II, and III vary between 25 to 40 %. The majority of inhibitory interneurons express paired box gene 2 (Pax2), which can be used to identify the distribution of both GABAergic and glycinergic interneurons in the dorsal horn (Punnakkal et al., 2014; Larsson, 2017). A dense network of inhibitory axons in superficial laminae, providing inhibitory control of somatosensory synapses, was described using staining against inhibitory neurotransmitter transporters vesicular

GABA/glycine transporter (VGAT) and neuronal glycine transporter (GLYT2) and an enzyme synthesizing GABA glutamate decarboxylase (GAD) (Todd, 2010).

2.3.2. Ascending pain pathways and descending modulatory tracts

Projection neurons transmit nociceptive information from the dorsal horn of the spinal cord to the higher brain areas via ascending pathways. Proper function of nociceptive pathways is crucial for the normal perception of nociceptive stimulation and damage to ascending nociceptive pathways can result in neuropathic pain. Axons of the projection neurons at first lead to the contralateral segment of the spinal cord, where they form ascending tracts. Five major ascending pathways combine nociceptive, thermosensitive, and wide-dynamic range projection neurons from the dorsal horn and project mainly into the thalamic nuclei and the brainstem.

The major ascending nociceptive pathway – the spinothalamic pathway – is formed by two spinothalamic tracts, transmitting information from the spinal cord to thalamic nuclei, more specifically to the lateral and medial nuclear groups. The spinothalamic pathway integrates nociceptive information from projection neurons of lamina I and V (Patestas et al., 2006). Thalamic nuclei are the main nociceptive relay in the central processing of the nociceptive information and process sensory discriminative and affective motivational components of pain (Patestas et al., 2006).

Projection neurons in the spinomesencephalic tract form synapses in the periaqueductal gray and the reticular formation of the midbrain. It is a direct pathway important in the inhibitory control of the nociception by activation of descending inhibitory pathways (Willis et al., 1997). Projection to the periaqueductal gray contributes to the aversive behavior (Willis et al., 1997).

Processing and modulation of the nociceptive information in higher brain regions involve descending pathways, projecting from the brain to the dorsal horn, that either facilitate or inhibit synaptic transmission in the dorsal horn. Main control over all descending pathways comes from the periaqueductal grey of the midbrain (Steeds, 2016). Descending pathways can be classified on basis of the neurotransmitter they express; monoamine-expressing pathways are serotonergic, noradrenergic, and dopaminergic, non-monoaminergic pathways include GABAergic and glycinergic descending projections (Antal et al., 1996; Millan, 2002; Todd, 2010).

Nociceptive information is subsequently relayed to the cortical regions of the brain, responsible for the conscious perception of the pain. Cortical regions most important in pain perception are the primary and secondary somatosensory cortex, insular cortex, anterior cingulate cortex, and prefrontal cortex (Treede et al., 1999).

3. Chronic and maladaptive pain

3.1. Neuroinflammation

Inflammation under physiological conditions represents a protective mechanism, characterized by increased blood perfusion of the damaged tissue and modulation of the capillary permeability that facilitates the migration of leukocytes into the inflamed tissue (Holzer, 1998). Migrating leukocytes provide an essential microbial and immunological defense and facilitate wound healing. This precise and fast action would not be possible without coordination by a number of different molecules such as cytokines, free radicals, eicosanoids, and transcription factors. At the same time these molecules, and cytokines, in particular, facilitate pain emerging from the damaged peripheral nervous system (PNS) at the site of the injury (Watkins et al., 2002). A rapidly growing body of evidence highlights the importance of the immune system in not only the initiation but also the maintenance of pathological pain by the potentiation of the activity of nociceptive sensory neurons. Moreover, peripheral inflammation accompanied by cytokines activating nociceptors can evolve into activation of microglia and astrocytes in the CNS and induce central sensitization. For this reason, neuroimmune interactions represent a significant factor in nociceptive processing, and the reaction of the immune system can be a driving force of the developing hypersensitivity and chronic pain (Ji et al., 2014).

Progression of neuroinflammation is accompanied by infiltration of leukocytes into the nervous system. Neutrophils infiltrate CNS in the initial hours of neuroinflammation, followed by macrophages in a matter of days and T cells in days or weeks after the trafficking starts (Costigan et al., 2009; Old et al., 2014). Infiltrating leukocytes amplify neuroinflammation by activation of glial cells – Schwann cells in the affected nerve, followed by satellite glial cells in the DRG and finally astrocytes, microglia, and oligodendrocytes in the CNS (Ellis et al., 2013). Similar to the inflammatory process in peripheral, non-neuronal tissue, neuroinflammation features increase the permeability of

the blood-brain barrier to allow leukocyte infiltration. CNS infiltration by leukocytes is of course easily evident and well described in models of the spinal cord or spinal nerve injury (Kigerl et al., 2009), but can accompany less obvious painful conditions, such as chemotherapy-induced peripheral neuropathy (Adamek et al., 2022). Rapid progression of neuroinflammatory mechanisms indicates that neuroinflammation stands at the establishment of chronic pain and presents a target for pain management in the early phase of chronic pain.

Besides injury of the PNS, neuroinflammation is associated with neuropathic pain in various chronic illnesses, for example, human immunodeficiency virus (HIV) infection. The development of chronic pain in HIV-positive patients has been linked to an increase in proinflammatory cytokines and activation of astrocytes in the spinal cord dorsal horn (Shi et al., 2012). Another example is chronic muscle pain endured by patients with fibromyalgia. In the case of fibromyalgia, chronic neuroinflammation of small nerve fibers leads to the development of neuropathic pain (Üçeyler et al., 2013).

3.2. Neuropathic pain

Neuropathic pain originates from damage to the PNS or CNS. Neuropathic pain does not develop in a response to a noxious stimulus like nociceptive pain; it is caused by a lesion or a disease of the somatosensory nervous system. Disorders of the peripheral nervous system leading to neuropathic pain range from CIPN, diabetic neuropathy, postherpetic neuralgia, nerve lesions, and trigeminal neuralgia to postamputation pain. Neuropathic pain can also manifest through a projected pain symptom induced by disorders such as stroke, spinal cord injury, and multiple sclerosis; therefore, the anatomical location of neuropathic pain can vary substantially based on the underlying disorder and can be perceived by a patient in peripheral tissues even when damage is limited to the central nervous system. Neuropathic pain is a chronic maladaptive condition, often associated with abnormal sensation; despite the diversity of conditions causing neuropathic pain, patients often share common qualities of neuropathic pain including burning or coldness, shooting or stabbing sensation, tingling, and numbness. Neuropathic pain is also associated with pain in an area with complete or partial sensory loss, increased pain after a repetitive stimulation, or pain persisting after the stimulation ceased (McMahon et al., 2006).

Two prominent symptoms typical of neuropathic pain are allodynia and hyperalgesia:

Allodynia is a condition defined by a feeling of pain elicited by a stimulus, that does not normally cause pain. A most common example of allodynia is a sunburn, a skin burn that reacts to innocuous stimuli with pain perception that is induced primarily by peripheral changes in the damaged tissue. Allodynia can be caused by more severe neuropathic conditions such as diabetes-induced neuropathic tactile allodynia or postherpetic neuralgia, where the changes are mostly due to pathological changes in the peripheral nerves and central sensitization processes. Further classification of allodynia is dependent on underlying peripheral or central sensitization and includes tactile and thermal allodynia, dynamic or static allodynia, and movement allodynia, describing pain caused by normally non-painful movement of joints or muscles. Allodynia can stem from damage to the peripheral nervous system or can develop in CNS via the process of central sensitization.

Hyperalgesia is an exaggerated sensitivity to a normally painful stimulus. This hypersensitivity of a peripheral sensory system occurs in response to infection or disease but can develop from damage or a lesion affecting the peripheral nervous system. As in allodynia, hyperalgesia can originate in the periphery by a local sensitization of nociceptors, but central sensitization may also play an important part in its development.

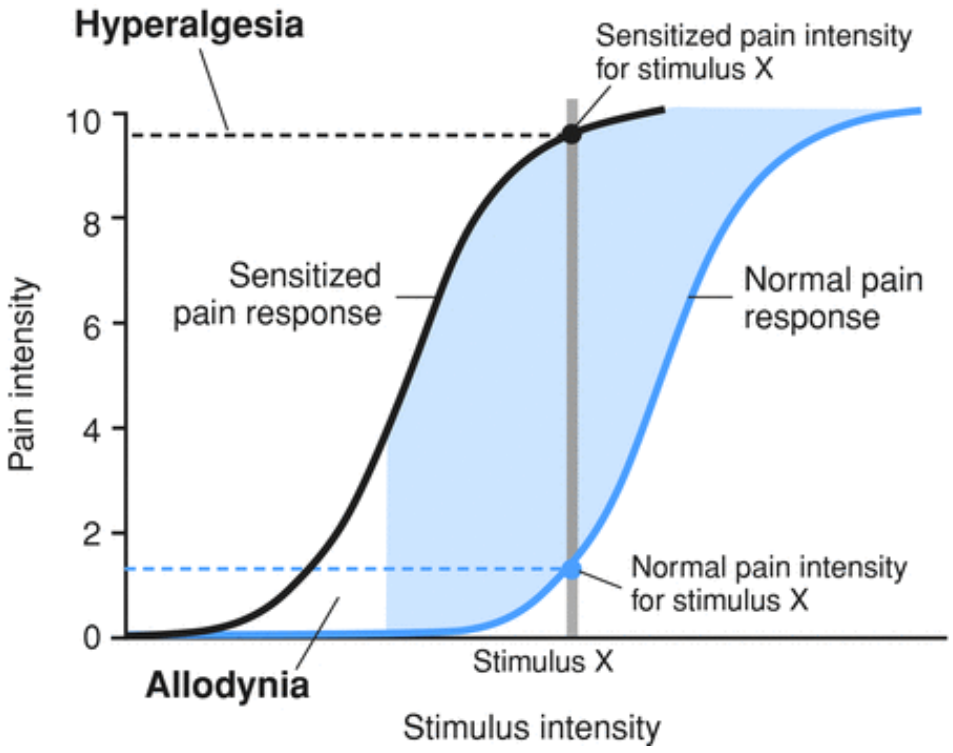

Figure 3.1.: **Graphical representation of pain threshold shift after injury.** Sensory inputs up to the normal pain threshold evoke non-painful sensory signalization. Injury in this representation shifts the normal pain threshold left, causing allodynia, triggering nociceptive stimulation at normally non-painful stimulus intensity. The leftward shift of pain threshold also leads to an increase in pain intensity evoked by painful stimuli, or hyperalgesia. Adapted from (Galvagno et al., 2011).

Neurons and synapses are plastic and are subject to constant modifications. This plasticity of expression and chemical profiles, intracellular transport, membrane composition, and structure allows neurons and synapses to change function in response to stimulation and extracellular signals. For example, phosphorylation of receptors and ion channels on the membranes of the nociceptors is a common and fast-acting mechanism of nociceptive pathway modulation, that can occur on peripheral nociceptors as well as in second-order dorsal horn neurons. Modulation of the primary nociceptors can often increase their excitability by shifting their depolarization threshold, facilitating the process of peripheral sensitization (Woolf et al., 2000).

Peripheral sensitization often occurs after a tissue injury or is triggered by an inflammatory response, by inflammatory mediators and extracellular signaling molecules released by the immune and the injured cells. Some of the most common molecules with the ability to sensitize peripheral nociceptors are neurotransmitter serotonin (Rueff et al., 1992), proinflammatory tissue hormone prostaglandin E_2 (Ma et al., 2017), ATP (Bardoni et al., 1997), and growth factor NGF (Stein et al., 2006). Most of the known sensitizing agents transduce their signals to intracellular signaling pathways via activation of G-protein coupled receptors (GPCRs) or tyrosine-kinase receptors. After activation, GPCRs and tyrosine-kinase receptors stimulate the activity of different kinases, such as protein kinase A (PKA), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K). Various ion channels and receptors can be phosphorylated as a result of this signaling, leading to a modulation of their function or properties (Woolf et al., 2000; Zhu et al., 2007 a; Zhang et al., 2011).

Central neuropathic pain can develop as a consequence of peripheral neuropathic pain or can be caused by lesions in CNS (for example multiple sclerosis or spinal cord injury); overall, while mechanisms of development of peripheral and central neuropathic pain

differ, they share major symptoms. Peripheral neuropathic pain can develop after localized insult to the PNS or can be spurred by damage of unmyelinated C fibers and thinly myelinated Aδ fibers, induced by metabolic damage (diabetic painful neuropathy or vitamin B12 deficiency), toxins (alcoholic neuropathy), or medication (CIPN). Inflammatory response involving cytokines and other inflammatory mediators can also result in peripheral neuropathic pain, caused by post-herpetic neuralgia or HIV (Bhangoo et al., 2007). Metabolic or inflammatory damage of the peripheral nerves results in substantial changes in fiber density and increased neuronal hyperexcitability (Rowbotham et al., 1996; Ochoa et al., 2005; Reichling et al., 2009; Ratté et al., 2016). Localized trauma to the axons of the nociceptors such as compression or hypoxia, but also inflammation or overstimulation can lead to fiber degeneration as well as changes in protein expression (Novakovic et al., 1998). These changes often lead to an ectopic firing–action potential generated within the nociceptive pathway, which does not originate in peripheral terminals.

Peripheral neuropathic pain can originate from damage to nociceptive neurons in DRG. Primary afferent neuronal bodies in DRG and trigeminal ganglia can be damaged by chemical, mechanical, or other excitotoxic insults in a similar fashion to their peripheral axons. Maladaptive changes in DRG can lead to a change in the membrane protein composition, resulting in altered properties of the first nociceptive synapse in the spinal cord dorsal horn (SCDH). Ultimately these changes form a maladaptive mechanism leading to a generation of inappropriate signals from the nociceptors (Study et al., 1996; Sandkühler, 2009).

Central neuropathic pain is a syndrome caused by a lesion or damage to the CNS. While peripheral neuropathic pain often involves both peripheral and central mechanisms of neuropathic pain, central neuropathic pain can develop as a result of a central sensitization originating in the CNS.

3.2.1. Central sensitization

IASP defines central sensitization as “increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input”. Simply put, central sensitization of spinal or supraspinal nociceptive pathways occurs after intense or repeated stimulation and can become maladaptive if the stimulation is persistent. Increased neuronal activity can be attributed to an increased synaptic efficacy between

primary nociceptive neurons and spinal cord dorsal horn neurons. Strengthening of the synapse allows for activation of second-order neurons in the spinal cord by normally subthreshold presynaptic inputs. Synaptic facilitation in central sensitization is similar to the mechanism of long-term potentiation and contributes to the development of primary hyperalgesia (Sandkühler et al., 2012).

In general, mechanisms involved in central sensitization include phosphorylation of pre- and postsynaptic receptors, modulation of membrane protein trafficking, and changes in expression. Excessive activation of the nociceptors and dorsal horn neurons results in a rise in the intracellular Ca^{2+} levels, which triggers Ca^{2+}-dependent intracellular signaling pathways and kinases. Activation of various kinases in the nociceptors - including PKA, PKC, PI3K, and others - potentiates the excitatory neurotransmitter release from presynaptic endings or modulates the composition of vesicles released to the synaptic cleft. On the postsynaptic neuron, targets of the phosphorylation involved in central sensitization include ionotropic and metabotropic glutamate receptors (Hildebrand et al., 2016; Choi et al., 2017). Evidence for the phosphorylation of these kinases comes from several animal models of pain such as PIPN (Dina et al., 2001) and nerve injury (Miletic et al., 2004; Miyabe et al., 2005).

An increase in postsynaptic receptors' conductivity and excitability is further achieved by alterations in membrane trafficking and expression. Phosphorylation of AMPA receptors increases their trafficking and incorporation into the postsynaptic plasma membrane. Changes in expression of ion channels, scaffolding proteins, glutamate transporters, etc. represent a more permanent modulation of a nociceptive synapse that is usually described as a late phase of central sensitization and further contributes to the alterations in glutamate homeostasis and facilitation of synaptic inputs.

3.2.2. Wind-up

Wind-up is a short-term homosynaptic mechanism facilitating synaptic transmission, that plays a role in the early phase of central sensitization. Repetitive synaptic activity, encoded by action potentials at frequencies above the critical rate, lead to an increase in pain intensity over time. Unmyelinated C group nerve fibers activated by a repeated stimulation release glutamate and neuromodulators including SP and CGRP to the synapse with the spinal lamina I neurons (Mendell, 1984; Battaglia et al., 1988).

Temporal summation of these inputs together with an increase in excitability contributes to the synaptic potentiation and development of secondary hyperalgesia.

3.2.3. Disinhibition

GABAergic and glycinergic interneurons in the spinal cord dorsal horn provide an inhibitory control of nociceptive transmission, crucial for normal signal transduction. Disinhibition -a loss or a disbalance in the inhibitory control - often accompanies the increase in the membrane excitability and synaptic potentiation and plays an important role in neuropathic and inflammatory pain (Zeilhofer, 2005). Some synapses that are under normal conditions silent, such as Aβ-low-threshold mechanoreceptors converging with nociceptive pathways to wide-dynamic range projection neurons in dorsal horn, can become active if the inhibition is lost due to disinhibition. This leads to an activation of pain pathways by normally mechanosensitive fibers, contributing to the development of a secondary hyperalgesia, characterized by an increase in sensitivity to stimulation of the tissue surrounding the site of injury (Woolf, 2011). The phenomenon of this heterosynaptic facilitation was shown for example as an increase in pinprick sensitivity in the area surrounding the site of intradermal capsaicin injection, mediated by A-fiber neurons (Ziegler et al., 1999). Inflammation of a hind paw induced by injection of the complete Freund's adjuvant increased activity of silent synapses between A-fibers and lamina I neurons, which contributes to secondary hyperalgesia (Torsney, 2011).

4. TRPV1 in pain processing

4.1. TRPV1 receptors and their structure

TRPV1 was the first isolated member of the TRPV proteins family and is most commonly associated with the regulation of body temperature and nociception. TRPV1 was cloned and characterized for the first time in 1997 (Caterina et al., 1997). TRPV1 is also known as vanilloid receptor 1 (VR1) or capsaicin receptor for its most well-known agonist capsaicin, the pungent active component of chili peppers.

TRP channels are nonselective cation channels formed by 4 subunits in either homo- or hetero-tetrameric conformations. Each subunit is formed by six putative transmembrane segments S1-S6. Cation influx is mediated through a reentrant loop, a hydrophobic loop between the S5 and S6 helix (Owsianik et al., 2006). Both N- and C- terminal domains of

the TRP receptor are cytosolic. The C-terminal domain contains the TRP box, important for the tetramerization of the protein (García-Sanz et al., 2004).

TRPV1 is a homo-tetrameric protein with a molecular weight of 92 – 95 kDa (Caterina et al., 1997). Proline-rich ankyrin repeat domain at the hydrophilic N-terminal domain of TRPV1 interacts with the cytoskeleton and contains the arginine R114 important for the binding of capsaicin.

The C-terminal domain of TRPV1 contains phosphorylation sites of PKC, PKA, and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and a Walker motif, the binding site of ATP and Phosphatidylinositol 4,5-bisphosphate (PIP_2). PKC was shown to be a crucial activator of TRPV1 in pain states (Premkumar et al., 2013). Phosphorylation of TRPV1 by the PKC potentiated TRPV1 and increased its sensitivity to low-concentration endogenous agonists, in a concentration that would not activate TRPV1 under normal conditions (Dray et al., 1988, 1992; Ahern, 2003; Premkumar et al., 2013). PKA-mediated phosphorylation of TRPV1 activates TRPV1 in inflammatory hyperalgesia (Jeske, 2008). Both kinases (PKA and PKC) require A-kinase anchoring protein (AKAP), scaffolding protein binding kinases and receptors to a signaling complex, allowing phosphorylation of TRPV1 after activation of kinases. The role of AKAP was demonstrated using an AKAP antagonist; administration of the antagonist reduced TRPV1-mediated, PKA-dependent thermal sensitization and decreased phosphorylation of TRPV1 (Jeske, 2008).

Intracellular Ca^{2+} modulates TRPV1 activity, especially via CaMKII. Phosphorylation of TRPV1 mediated by CaMKII is a necessary step before the receptor activation by capsaicin. CaMKII together with phosphatase calcineurin exerts regulatory control over TRPV1 by balancing phosphorylation and dephosphorylation orchestrated by Ca^{2+} influx (Jung et al., 2004; Nagy et al., 2014).

PI3K is another kinase important in TRPV1 modulation, reported to sensitize TRPV1 in both neuropathic and inflammatory pain. PI3K-induced sensitization of TRPV1 in PIPN and underlying mechanisms were studied by our group and their detailed description can be found in the Discussion (Adamek et al., 2019, 2022). Stimulation of PI3K and its signaling pathway by NGF sensitized capsaicin-mediated TRPV1 activation, by inducing rapid trafficking of the TRPV1 channel to the membrane (Zhang et al., 2005; Stein et al., 2006; Zhu et al., 2007 b).

4.2. TRPV1 in nociception

TRPV1 is a polymodal receptor – activation of TRPV1 can be achieved through both physical and chemical stimuli and can arise from the environment, or as a response to the changes in homeostasis and endogenous agonists. In the context of nociception, TRPV1 is expressed by peripheral endings of nociceptive C and Aδ fibers, but also at the presynaptic ending in the dorsal horn. Studies of rat and human orthologues of TRPV1 found high expression of TRPV1 in DRG and trigeminal ganglia and a low expression throughout other CNS structures (cerebellum, hippocampus, and frontal cortex) (Hayes et al., 2000).

Evidence for the TRPV1 in C and Aδ fibers comes from the studies that observed the expression and immunoreactivity of TRPV1 in small and medium-sized neurons within DRG and trigeminal ganglia (Caterina et al., 1997; Guo et al., 1999). High immunoreactivity of TRPV1 positive presynaptic endings was observed in lamina I-II of the dorsal horn, as is also evident from autoradiography on human post-mortem samples, which revealed a high abundance of TRPV1-positive terminals in the dorsal horn (Szallasi et al., 1994). While TRPV1 can be identified across all spinal cord segments, autoradiography revealed the highest densities in the lumbar segment, almost double compared to cervical and thoracic segments (Szallasi et al., 1995). Variation of the density of TRPV1-immunoreactive fibers and buttons in the dorsal horn was observed inside the lumbar segment as well, with a progressive increase from segments L4 to L6 (Hwang et al., 2003).

During development TRPV1 receptors in the spinal cord undergo significant changes in terms of both receptor density and localization. Expression of the spinal TRPV1 in the early neonatal stage at postnatal day 2 (P2) in rats is three-fold higher than in adult animals (Acs et al., 1994). Distribution of the TRPV1 between laminae of the dorsal horn shifts during embryonic and postnatal development. TRPV1 was found to be present only in lamina I between embryonic day 16 and P2. The first significant expression of TRPV1 in lamina II can be observed around P10 and continues to develop and increase in density up to P20 (Guo et al., 2001). The adult-like distinctive pattern of TRPV1 in lamina I and II_i recognizable by immunohistochemistry can be first observed around P30 (Guo et al., 2001).

TRPV1 receptors in the dorsal horn colocalize with peptidergic afferent neuron terminals, positive for SP and CGRP (Saria et al., 1986). High levels of colocalization of TRPV1 and SP were shown especially in lamina I of the L6 segment (Hwang et al., 2003). In lamina II_i TRPV1 receptors in lamina II_i coexpress with $P2X_3$ – purinoceptor related to nociception and pain – and with IB4, glycoprotein binding lectin (Guo et al., 1999). These coexpression patterns highlight the importance of TRPV1 in nociceptive signaling, as approximately 80% of lamina I projection neurons express NK1R – receptor for SP – which is considered to be important in thermal hyperalgesia (Mantyh et al., 1997). TRPV1 positive glutamatergic afferent neurons form synapses with NK1 and NMDA positive projection neurons in lamina I and transduce a signal through a monosynaptic pathway directly from peripheral endings to the lateral parabrachial nucleus, a so-called hub for pain and aversion (Hwang et al., 2003, 2004).

4.2.1. TRPV1 activators and modulators

TRPV1 is a nonselective cation channel, with a preference for divalent cations, with highest permeability for Ca^{2+} ions, followed by Mg^{2+} ions and monovalent cations ($Ca^{2+} > Mg^{2+} > Na^{+} \approx K^{+} \approx Cs^{+}$). TRPV1s high permeability for Ca^{2+} ions leads to a membrane depolarization upon activation and a steep increase in the intracellular Ca^{2+} concentration (Oh et al., 1996; Caterina et al., 1997). TRPV1 activation-induced increase of the intracellular Ca^{2+} concentration leads to a neurotransmitter release independent of an action potential (Medvedeva et al., 2008).

At first, most of the TRPV1 research focused on activators and modulators of TRPV1 in peripheral tissue. Activators of TRPV1 include both exogenous and endogenous compounds and noxious heat at over 43 °C at the physiological pH (Tominaga et al., 1998, 2004). Moreover, several endogenous compounds, as well as temperature, can sensitize TRPV1, facilitating the channel opening at a low change in membrane potential (Nilius et al., 2007). Well-known exogenous agonists of TRPV1 are capsaicin and resiniferatoxin – ultra-potent capsaicin analog (Szolcsanyi et al., 1990). Other exogenous agonists are eugenol and guaiacol, organic compounds found in the clove oil; piperine, alkaloid found in black pepper, or olvanil and nuvanil, synthetic vanilloids (Szallasi et al., 1999).

The majority of endogenous TRPV1 agonists can be classified as N-Acyl amides and fatty acid metabolites. Lipidic neurotransmitter N-arachidonoylethanolamine

(anandamide) acts as an agonist of both TRPV1 and cannabinoid receptor 1 (CB_1) (Devane et al., 1992; Zygmunt et al., 1999; Tognetto et al., 2001). Anandamide is synthesized from its precursor arachidonoylphosphatidylethanolamine (20:4-NAPE) through various metabolic pathways (Uyama et al., 2013). Synthesis and degradation of anandamide were observed in both DRG and spinal cord (Carrier et al., 2004; Van Der Stelt et al., 2005; Vellani et al., 2008; Varga et al., 2013), and application of 20:4-NAPE increased anandamide production in cultured DRG neurons, in concentration and temperature-dependent manner (Varga et al., 2013; Nerandzic et al., 2017).

More endogenous agonists, products of lipoxygenases, are unsaturated N-acyldopamines first identified in samples isolated from bovine striatum. These TRPV1 ligands include N-arachidonoyldopamine (NADA) and N-oleoyldopamine (OLDA). While low concentrations of OLDA can induce potent intracellular Ca^{2+} mobilization in TRPV1-expressing cells, it is only a weak ligand of CB_1 (Chu et al., 2003). OLDA is transported by the anandamide membrane transporter and its metabolic inactivation by fatty acid amid hydrolase is significantly slower than in the case of NADA (Chu et al., 2003). In behavioral experiments OLDA induced thermal hyperalgesia; OLDA proved to be more potent than capsaicin and OLDA-induced hyperalgesia lasted for a longer duration compared to the effect of capsaicin (Chu et al., 2003; Spicarova et al., 2009). Inflammatory mediators increase the sensitivity of TRPV1 to OLDA dramatically, hinting at a potentially prominent role of OLDA in the inflammation-induced thermal and mechanical hypersensitivity (Uchytilova et al., 2021).

4.2.2. TRPV1 desensitization and antagonists

Receptor desensitization is caused by a change in receptor or channel conformation and leads to a cessation of agonist-induced signaling. Desensitization can be permanent or transient and decreases sensitivity to the agonist. Functionally, desensitization is a negative feedback loop that can have a protective role against prolonged and potentially harmful intracellular signaling. In the case of TRPV1, high permeability for Ca^{2+} leads to a robust increase in intracellular Ca^{2+} which can eventually lead to a Ca^{2+} overload and cell death (Touska et al., 2010), as was demonstrated on nociceptors using capsaicin (Lawson, 1987). Acute desensitization of TRPV1 is triggered by Ca^{2+} influx itself, as intracellular Ca^{2+} activates calcium-dependent phosphatases, that dephosphorylate TRPV1 to reduce its activation (Zhang et al., 2008). Moreover, Ca^{2+} ions bind to calmodulin, creating a Ca^{2+}-calmodulin complex, that acts as a negative modulator of

PKA, PKC, and other kinases (Lishko et al., 2007). Conformational change evoked by agonist-induced desensitization of TRPV1 leads to desensitization lasting approximately 20 minutes (Mohapatra et al., 2003).

TRPV1 activation induced by capsaicin, or its analogs can have local acute analgesic effects mediated by receptor desensitization or can even lead to degeneration of TRPV1-positive nociceptive C-fibers (Simone et al., 1989; Pospisilova et al., 2006). Two potential analgetic approaches, aimed at the properties of TRPV1 in nociceptive pathways have been proposed. A pharmacological interaction with the spinal TRPV1 could provide potent analgesia, without compromising normal thermal sensitivity (Fischer et al., 2013), or long activation and resulting Ca^{2+} overload of neurites could lead to long-lasting analgesia by ablation of nociceptive central terminals (Jeffry et al., 2009). Capsaicin-induced desensitization found its use in modern medicine through capsaicin patches and gels, providing effective local analgetic effects and attenuation of secondary hyperalgesia (Haanpää et al., 2012; Henrich et al., 2015).

Antagonists of TRPV1, ligands that block the activity of TRPV1, have been extensively studied for their potential to mitigate neuropathic and inflammatory pain. Their use in medicine is complicated by difficulties of systemic administration, as they affect normal thermal sensation and regulation of body temperature (Gavva et al., 2007; Xia et al., 2011). This body temperature regulation is controlled by visceral, tonically active TRPV1, continuously suppressing body temperature (Gavva, 2008). Thanks to the modality of a specific agonist of TRPV1 studies on newly developed antagonists of TRPV1 report no effect on body temperature regulation (Joseph et al., 2019) but concerns of negative side effects such as a danger of heat burn for patients with TRPV1 systemic antagonists due to on target effect of change threshold for noxious heat remain.

4.2.3. Evidence for the role of TRPV1 in neuropathic pain

Spinal TRPV1 receptors were shown to play a major role in chronic and neuropathic pain states and their modulation often represents a crucial step in the development of the pain states. Activity or a concentration of a number of the TRPV1 modulators discussed in the previous chapters was shown to be elevated in DRG and spinal cord in chronic and neuropathic pain models. TRPV1-induced increase in the release of neurotransmitters also contributes to the development of chronic pain by a release of CGRP, a neuropeptide linked to chronic and inflammatory pain (Loyd et al., 2012).

Recent studies highlight a prominent role of TRPV1 expressed in DRGs in the development of PIPN (Li et al., 2015). Peripheral neuropathy and acute pain syndrome are major adverse effects of paclitaxel and underlying mechanisms are poorly understood (Reeves et al., 2012; Boyette-Davis et al., 2018). A study conducted in our laboratory in collaboration with Dr. Dougherty`s team showed that paclitaxel leads to direct activation of toll-like receptor 4 (TLR4) on peripheral sensory and spinal neurons. TLR4 activated by paclitaxel subsequently leads to the sensitization of TRPV1, which was demonstrated as an increase in the capsaicin-mediated response in acute spinal cord slices of rats and mice and neuronal cultures of dissociated rat and human DRGs (Li et al., 2015). Moreover, the same study demonstrated that paclitaxel-induced hyperalgesia can be prevented or reversed both by intrathecal (i.t.) TRPV1 and TLR4 antagonists. A TLR4-induced mechanism in PIPN mediates both acute and long-term sensitization of TRPV1 and underlying intracellular pathways remain to be fully identified.

Streptozocin (STZ) induced model of diabetic peripheral neuropathy leads to a painful phenotype, characterized by hyperalgesia and increased release of CGRP from primary sensory neurons (Ellington et al., 2002). STZ-induced hyperalgesia was linked to increased activity and sensitization of TRPV1 in the DRG and spinal cord (Pabbidi et al., 2008 a; b). Later studies confirmed that PKA and PKC-induced modulation of TRPV1 and upregulation of TRPV1 expression in DRG and spinal cord play a major role in diabetic thermal hyperalgesia (Pabbidi et al., 2017) although not in mechanical allodynia; i.t. administration of potent TRPV1 agonist resiniferatoxin (RTX) was only effective in attenuation of thermal hyperalgesia and did not affect mechanical allodynia (Bishnoi et al., 2011).

Evidence for the role of TRPV1 receptors in central neuropathic pain comes from a model of contusive spinal cord injury (SCI). Hypersensitivity to mechanical and thermal stimuli after SCI was linked to an upregulation of TRPV1 expression in the DRG and increased sensitivity to capsaicin in DRG neurons from mice after SCI. TRPV1 antagonist attenuated the increased mechanical and thermal hypersensitivity (Wu et al., 2013). Similarly to central neuropathic pain, TRPV1 antagonist attenuated mechanical allodynia induced by peripheral nerve injury in models of chronic constriction injury (CCI) (Kim et al., 2012) and spinal nerve ligation (SNL) (Watabiki et al., 2011).

4.2.4. Modulation of TRPV1 in inflammatory pain

Peripheral TRPV1 receptors are sensitized by local inflammation, either directly by inflammatory mediators, or indirectly by changes in the inflammation-induced changes in the surrounding tissue. Such a change can be local acidity, induced as a result of inflammation, injury, or ischemia. Lower levels of pH sensitize the peripheral TRPV1 receptor to its agonists, some of which are produced and released during inflammation. Besides direct activation, inflammatory mediators can also promote the expression of TRPV1, initiate its trafficking to the plasma membrane or sensitize TRPV1 to other of its agonists. Typical inflammatory mediators modulating TRPV1 receptors are bradykinin and tumor necrosis factor α (TNFα). TRPV1 receptor was shown to be sensitized to low concentration of its endogenous agonists in the presence of bradykinin (Spicarova et al., 2009) and TNFα (Spicarova et al., 2010). Normally an ineffective dose of OLDA was able to potentiate glutamate release in neurons incubated in bradykinin or TNFα.

Protease-activated receptors 2 (PAR2) are GPCRs expressed by approximately 60% of DRG neurons from L4 to L6 DRGs. Their well-established role in inflammatory pain and high coexpression with TRPV1 in DRG provides an additional link between the central (spinal) TRPV1 receptor and inflammatory thermal hyperalgesia mechanisms (Dai et al., 2004). Activation of PAR2 leads to TRPV1 receptor sensitization mediated by phospholipase C-β (PLCβ), PKC, and PKA signaling (Amadesi et al., 2006). Activated PAR2 in the spinal cord potentiates synaptic activity and induces thermal hyperalgesia via central sensitization; TRPV1 antagonist SB366791 prevented enhanced PAR2-induced synaptic activity (Mrozkova et al., 2021).

Proinflammatory cytokines, such as chemokine (C-C motif) ligand 2 (CCL2), activate the PKC signaling pathway and thus promote the phosphorylation and subsequent activation and sensitization of TRPV1 in DRG and spinal cord neurons (Spicarova et al., 2014; Zhao et al., 2014). Moreover, CCL2-induced signaling leads to an increase in the TRPV1 receptor and $Na_v1.8$, the sodium channel linked to nociceptive transmission (Kao et al., 2012). Coexpression of CCL2 with TRPV1 was described in sensory neurons of DRG and astrocytes in the spinal cord dorsal horn. A previous study from our group shows that i.t. CCL2-induced central sensitization can be attenuated by the administration of TRPV1 antagonist SB366791 (Spicarova et al., 2014).

Carrageenan inflammatory pain model induces local thermal hypersensitivity. Intrathecal administration of RTX significantly reduced thermal hypersensitivity, highlighting the important role of TRPV1 in carrageenan-induced thermal hypersensitivity (Jeffry et al., 2009; Mishra et al., 2010). Another model of thermal hyperalgesia, Complete Freund's adjuvant (CFA) induced inflammatory pain model, showed a significant attenuation of thermal hyperalgesia in TRPV1 knock-out mice (Caterina et al., 2000).

5. Animal models of neuropathic pain

Several animal models of neuropathic pain are widely accepted as pivotal tools to study mechanisms of neuropathic pain in humans and optimize chronic pain management. The diverse etiology of neuropathic pain in patients requires numerous animal models mimicking different mechanisms and manifestations of neuropathic pain. Most of the neuropathic pain models derive from nerve injury-induced neuropathic pain. This chapter will briefly summarize CCI and PIPN.

CCI is a model of a peripheral mononeuropathy, developed by Bennett and Xie (Bennett et al., 1988) and later improved by the use of loosely constrictive chromic gut ligatures which produce neuropathic pain without the epineural blood flow retardation (Maves et al., 1993). CCI is one of the most commonly used models of neuropathic pain, developed for both rats and mice. CCI is produced by a chronic constriction of the sciatic nerve, placing three loose ligatures proximal to the sciatic trifurcation around the sciatic nerve. Behavioral changes observed in CCI include mechanical and thermal hyperalgesia and cold allodynia, and animals express signs of spontaneous pain – excessive licking, limping, guarding, etc. Painful behavior develops within a week after surgery and can persist for 7 weeks after (Bennett et al., 1988; Dowdall et al., 2005). Mechanisms of nerve injury in CCI include intraneural edema, focal ischemia, and Wallerian degeneration. CCI induced a rapid increase in CCL2 expression in the spinal cord and CCL2 plays a significant role in the development of CCI-induced neuropathic pain (Kwiatkowski et al., 2019).

Paclitaxel (PAC) is a commonly used chemotherapeutic, effective in the treatment of breast, ovarian, esophageal, and other types of cancer. However, the risk of the

development of PIPN and the chronic pain that accompanies PIPN represents a major drawback and a dose-limiting factor of PAC chemotherapeutic therapy (Boyette-Davis et al., 2018). PIPN has been reported to induce significant sensory neuropathy, manifested mostly in the distal extremities, with symptoms that include mechanical allodynia, spontaneous burning pain, and feeling of tingling and numbness (Dougherty et al., 2004).

Low doses of PAC in repeated applications have been reported to induce peripheral neuropathy and chronic pain symptoms without any observable motor impairment or system toxicity (Adamek et al., 2019). Neuroinflammation of the PNS and CNS has been observed in the PIPN model, leading to central sensitization of nociceptive pathways (Kalynovska et al., 2020; Meesawatsom et al., 2020). Mechanical and cold allodynia induced by PAC can persist 3 weeks after the last dose of PAC. Low penetration of the blood-brain barrier and observed accumulation of PAC in DRG leads to selective damage to PNS and DRG, hence causing peripheral neuropathy.

PIPN-induced neuroinflammation is associated with several inflammatory molecules and pathways, for example, chemokine CCL2. PAC administration increased the expression of CCL2 and C-C motif chemokine receptor 2 (CCR2) in DRG neurons and spinal astrocytes and the expression of CCL2 in the spinal dorsal horn (Zhang et al., 2013). Inflammatory mediators elevated by PAC treatment are potent sensitizing agents of DRG and spinal cord neurons and the TRPV1 receptor plays an important role in PAC-induced sensitization (Kao et al., 2012; Nagy et al., 2014; Spicarova et al., 2014; Malek et al., 2015). Understanding of how PAC leads to TRPV1 sensitization and central sensitization might lie in toll-like receptor 4 (TLR4) receptor-induced signaling pathways.

It has been established that PAC can activate TLR4 and induce macrophage activation similar to lipopolysaccharide (LPS) induced activation of TLR4, suggesting that both PAC and LPS initiate similar intracellular signaling pathways (Kawasaki et al., 2000; Byrd-Leifer et al., 2001; Guha et al., 2001). On DRG neurons, TLR4 shows a high level of colocalization with TRPV1. A growing number of studies show a major role of neuronal TLR4 in the regulation of TRPV1 signaling and sensitivity, in various inflammatory conditions including PIPN (Li et al., 2015; Adamek et al., 2019; Wu et al., 2019). Recent studies confirm a direct sensitization of TRPV1 by the PAC-induced activity of TLR4, leading to the development of hypersensitivity, which was attenuated using the TLR4 antagonist (Li et al., 2015).

TLR4-induced sensitization of TRPV1 is dependent on phosphorylation of TRPV1 receptor by serine/threonine kinases, especially the PI3K. PAC-induced activation of the PI3K pathway was demonstrated by an increase in phosphorylation of Akt kinase, downstream of PI3K (Adamek et al., 2019). PI3K signaling contributes to the TRPV1 sensitization by facilitation of TRPV1 receptor trafficking to the plasma membrane and by conversion of PIP_2 to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Cao et al., 2013). Our group also established a link between PAC-induced PI3K signaling, PIPN, and neuroinflammation in studies from 2019 and 2022 (Adamek et al., 2019, 2022), more detailed information can be found in the Results and Discussion sections.

6. Chemokines in neuropathic and inflammatory pain

Although the interaction between the nervous and the immune system is orchestrated by a vast number of cell lines, receptors, and messenger molecules, chemotactic cytokines or chemokines has been directly linked to nociceptor sensitization.

Chemokines belong to a family of small (8-12 kDa) signaling proteins. The main function of chemokines in the immune system is to attract immune cells towards the source of the chemokine, typically an inflamed tissue in case of the inflammatory chemokines. The highly conserved location of cysteine residues in the structure of chemokines serves as a basis for their classification into four subfamilies:

CC chemokines: the CC chemokine subfamily is characterized by two adjacent cysteines near the amino terminus. This subfamily contains 27 chemokines – CC chemokine ligands 1 to 28. The general function of the CC chemokines in the immune system is a migration of various cell types, including monocytes, NK cells, and dendritic cells. CCL2 (chemokine (C-C motif) ligand 2, also known as monocyte chemoattractant protein 1) is a member of the CC chemokines subfamily.

CXC chemokines: classification of the CXC chemokines family is based on two N-terminal cysteines separated by one amino acid (represented by “X” in the name of the subfamily). CXC chemokines subfamily contains 17 chemokines that bind to seven known CXC chemokine receptors. This subfamily is further divided into two groups;

CXC chemokines containing glutamic acid-leucine-arginine motif (ELR) before the first cysteine and CXC chemokines without ELR motif.

C chemokines: only two cysteines, one N-terminal and one downstream characterize the C chemokines subfamily. Two members of the C chemokines are XCL1 and XCL2

CX3C: the last subfamily contains a single chemokine discovered to date, named fractalkine or CX3CL1 and unlike the rest of the chemokines, CX3CL1 has three amino acids between the two cysteines (X3).

Injured or infected tissue produces high concentrations of inflammatory chemokines, released typically in response to stimulation by inflammatory cytokines (for example interleukin 1). An increasing concentration gradient of inflammatory chemokines guides leukocytes to the damaged tissue, recruiting cells like monocytes, T-lymphocytes, or neutrophils from the blood to the source of chemokines. The inflammatory response is further potentiated by chemokines by binding to the chemokine receptors on the immune cells and promoting their inflammatory response.

6.1 Chemokines in nervous systems

In general, chemokines signal through 20 distinct G protein-coupled chemokine receptors with a rhodopsin-like 7-transmembrane structure, located predominantly on the surface of leukocytes but also on a vast number of different cell types throughout the body. Common structural features are 1.) length of about 350 amino acids; 2.) short N-terminal domain; 3.) seven helical transmembrane domains with three intracellular and three extracellular hydrophilic loops; 4.) intracellular C-terminal domain containing phosphorylation sites. Most of the chemokines bind to so-called conventional chemokine receptors, with a few exceptions binding to four atypical chemokine receptors with distinct transduction pathways (Nibbs et al., 2013). The following description of chemokine receptors will focus on the structure and function of conventional chemokine receptors. The most common signaling pathway activated by the binding of a chemokine to the chemokine receptor starts with pertussis toxin-sensitive Gαi G-proteins and β-arrestins. Chemokines bind to their respective receptors via the extracellular loops and N-terminus of the receptor; upon binding, the N-terminus of the chemokine enters the

heptahelical bundle, which induces a conformational change, activating complex intracellular signaling pathways (Kleist et al., 2016).

Since chemokine receptors are expressed also on other non-leukocytic cell types, chemokines can regulate the biology of many other cell types. In addition to their actions in the inflamed tissue, chemokines are important for the proper maturation of leukocytes, play a role in angiogenesis, and are expressed by neurons, neural progenitors, and glial cells (Cartier et al., 2005). Chemokine receptors are expressed throughout the CNS – primary afferent sensory neurons for example express several chemokine receptors. Thus, it is highly likely that chemokines have other functions in the CNS besides coordination of the inflammatory reaction and can act directly on CNS and PNS neurons.

Direct action of chemokines on sensory neurons was in fact repeatedly observed; most interesting for the purpose of this review is the direct potentiation of the primary nociceptive neurons by CCL2. CCL2 is also referred to as monocyte chemoattractant protein 1 (MCP1) and belongs to a monocyte chemoattractant protein subfamily together with CCL7, CCL8, CCL13, and CCL12. The main function of chemokines of the MCP family is to attract different leukocytes upon binding to their respective receptors (Gouwy et al., 2004). In the site of inflammation, CCL2 attracts monocytes, memory T cells, and dendritic cells through binding to its receptor CCR2 and with a lower affinity through receptor CCR4. CCL7 and CCL8 are also ligands of the CCR2 receptor but bind to the receptor with a much lower affinity than CCL2 (Sozzani et al., 1994). CCL2 is located in chromosome 17 of the human genome and is 76 amino acids long with a weight of 11.025 kDa. In non-neuronal tissue, CCL2 is primarily secreted by monocytes, macrophages, and dendritic cells and its major inducer is a platelet-derived growth factor. In CNS and PNS the CCL2 chemokine can be expressed by neurons, microglia, and astrocytes in various anatomical regions, often as a marker of ongoing neuroinflammation. Increased CNS levels of CCL2 produced by microglia were described in epilepsy, after focal cerebral ischemia, in Alzheimer disease; increased expression in astrocytes was described in experimental autoimmune encephalomyelitis. In traumatic brain injury or cortical lesions increased levels of CCL2 correlate with an extent of macrophage infiltration (Hausmann et al., 1998).

6.2. Chemokine CCL2 in nociceptive transmission

Chemokine CCL2 gained considerable attention in chronic pain and neuroinflammation, reaching from activation of immune response in the CNS, sensitization of nociceptive pathways, to the modulation of opioid signaling. CCL2-CCR2 axis proved to be critical in the development of neuropathic pain. Numerous studies observed increased CCL2 levels in animal models of neuropathic pain, such as CCI (Zhang et al., 2006; Piotrowska et al., 2016; Kwiatkowski et al., 2019; Zajaczkowska et al., 2020) and PIPN (Zhang et al., 2013). Its role was further emphasized when a single i.t. application of CCL2 evoked hypersensitivity in rats and mice while having no effect on the sensory system after CCR2 mRNA silencing (Tanaka et al., 2004; Spicarova et al., 2014; Bégin-Lavallée et al., 2016). Furthermore, peripheral nerve injury-induced mechanical hypersensitivity was attenuated in CCR2 knockout mice, proving the pivotal role of CCL2 in neuropathic pain development (Abbadie et al., 2003).

The expression pattern of CCL2 in the spinal cord and DRG indicates its involvement in pain transmission modulation. The presence of CCL2 in DRG neurons is primarily bound to the nociceptive small and medium-sized neurons and colocalizes significantly with TRPV1 receptors, SP, and CGRP (Dansereau et al., 2008). In the spinal cord, increased levels of CCL2 were observed following spinal nerve ligation and CCI, most prominently in superficial layers of the dorsal horn (Zhang et al., 2006; Gao et al., 2009). Immunoreactivity of CCL2 in the spinal cord correlates with SP and CGRP axon terminals, indicating a possible transport of CCL2 from neuronal soma in DRG (Dansereau et al., 2008; Thacker et al., 2009). CCL2 released in the spinal cord dorsal horn can then act as a neuromodulator on spinal neurons and glial cells – release of synaptic vesicles containing CCL2 from primary nociceptive neurons has been observed in the CCI model, where CCL2 originated from TRPV1 positive fibers. In this study, CCI upregulated proinflammatory markers and promoted activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the dorsal horn, while i.t. CCR2 antagonist reversed these changes (Van Steenwinckel et al., 2011). Astrocytes are another source of chemokine release in the spinal cord, as astrocytes were shown to produce CCL2 upon activation by a TNFα (Gao et al., 2009). In the DRG, upregulation of both CCL2 and CCR2 protein was observed on day 7 after CCI; authors of the same study also

successfully attenuated CCI-induced mechanical and thermal hyperalgesia by i.t. CCR2 antagonist (Kwiatkowski et al., 2017).

While peripheral and central sensitization is crucial for the development of neuropathic and inflammatory pain, the amplified neuronal response is often caused by mechanisms involving glial cells. Chemokines have been shown to serve as mediators in neuron-glia communication, especially the CC chemokine family (Conductier et al., 2010). Their role in glial activation was observed in different pain models (Kwiatkowski et al., 2017; Chun et al., 2019) and microglial activation caused by cytokine release is one of the mechanisms underlying inflammation-induced central sensitization (Chun et al., 2019). CCL2 represents an important messenger in microglial activation; this was demonstrated by i.t. administration of CCR2 antagonist, which attenuated the release of other cytokines in the CCI model (Kwiatkowski et al., 2017). The importance of the microglial component was also demonstrated by the administration of the minocycline, a blocker of microglial activation, which attenuated CCL2-induced hyperalgesia (Huang et al., 2014 a).

7. Opioids

Opioids – agonists of opioid receptors – are a broad group of substances probably best known for their pain-relieving and anesthetic effects. Opioids range from naturally occurring compounds found in the poppy plant, endogenous ligands of the opioid receptor such as nociceptin, to extremely potent synthetic opioids like fentanyl. The first documented medical use of opium dates back more than 5000 years and its analgesic and anti-diarrheal properties were well described in ancient Greek medicine. Opioids are regarded as a gold standard of analgesics in acute pain treatment, providing immediate relief of severe acute pain. Opioids are frequently used for short-term pain management after surgery or trauma, where the risk of developing dependence is relatively low.

While opioids have other effects on human physiology, the most significant focus in both basic and clinical research remains analgesia. Probably the best known and most extensively studied analgesic drug is opiate morphine. A scientific study of morphine can be even dated back to Darwin, who examined the effect of morphine on plants. As with nociception, a study of analgesia represents a complex problem; because the study of analgesia requires the presence of pain, a suitable animal model of pain is of utmost

importance. Analysis of opioid analgesia is complicated by several factors, such as the severity of the nociceptive stimulus. Pain-relieving doses of any analgesic vary with the intensity of perceived pain, and not all drugs that relieve mild pain are potent enough to provide satisfactory pain relief in models of severe pain. The next factor to consider is the quality or type of pain. Different types of pain typically differ in their sensitivities to various analgesics. Neuropathic pain is probably the best example; most traditional analgesics usually fail to deliver effective analgesia in neuropathic pain, while different classes of drugs that are not classical analgesics can be effective (e.g. tricyclic antidepressants and anticonvulsants) (Dworkin et al., 2003). In the case of morphine analgesia specifically, while morphine is a potent analgesic, its dosage varies among species, or even strains of laboratory animals, to an enormous extent (Kest et al., 1998). Similar to nociception, significant differences in morphine sensitivity within species can be observed between sexes (Mogil et al., 2010). Finally, the last complication in a study of analgesia is the subjective nature of pain. Animal models provide important insight and understanding of nociception and analgesia, it is limited in comparison to clinical situations with the subjective perception of pain.

Increased prescription of opioids in past decades in the United States of America lead to a so-called “opioid crisis” with devastating consequences rising from opioid misuse. While opioids are effective in treating acute pain, they are less effective in treating chronic and neuropathic pain. Patients prescribed opioids to treat chronic pain experience long-term exposure to opioids, which can lead to the development of opioid tolerance. Low effectiveness in chronic and neuropathic pain combined with opioid tolerance results in increased doses of opioids or administering opioids with higher potency to re-establish sufficient analgesia. A cycle of escalating doses to combat tolerance inevitably increases the risks of adverse side effects and addiction, which often results in the abuse of opioid substances (Okie, 2010).

Highly addictive opioids are accompanied by a long list of adverse effects and a high risk of overdose – opioid overdose in the United States claimed more than 68,000 lives in 2020, a disturbingly steep increase from 4,000 deaths reported in 1999 (Okie, 2010; NIDA, 2022). Addiction combined with physical dependence and tolerance – a process of neuroadaptation, which results in a reduction of drug effects and the necessity to increase the dose of a medication over time to achieve the same effect – increases the likelihood of opioid overdose compared with other prescription medications. In fact, opioid

dependence is the main risk factor for opioid overdose, followed by intravascular injection of opioids, resumption of opioid use after a period of abstinence, and using opioids in combination with alcohol, benzodiazepines, or cocaine (WHO, 2021). Over-activation of opioid receptors, distributed throughout various systems and organs, typically leads to severe respiratory depression, which can lead to hypoxia if not treated immediately. If not lethal, intoxication by opioids and subsequent hypoxia can lead to permanent brain damage. Opioid overdose can be treated by providing basic life support, including oxygen to prevent hypoxia and opioid receptor antagonist naloxone (De Caen et al., 2015).

Other frequent adverse effects include nausea and vomiting, itching, constipation, respiratory depression, and increased pain sensitivity commonly known as opioid-induced hyperalgesia (OIH). OIH was first described as a side effect of long-term use of opioids and can develop after administration of various opioids, as described in studies using morphine, heroin, and fentanyl. For long time mechanisms causing OIH has been difficult to distinguish, due to their diversity and the ability of opioids to cause hyperalgesia through action on both the peripheral and central nervous system. In general, OIH develops when activation of opioid receptors causes long-lasting neuromodulation, which after initial analgesia later leads to enhanced nociception. Pain from OIH often manifests as generalized or diffuse pain with vague localization, not always confined to the original site of injury or disease. This makes the diagnosis, treatment, and consequently also the study of OIH very challenging.

7.1. Opioid receptors

Opioids act through interaction with opioid receptors, which belong to a family of G protein-coupled receptors (GPCRs). In short, GPCRs are characterized by an extracellular N-terminus followed by a seven-transmembrane α-helical structure with three extracellular and three intracellular loops and an intracellular C-terminus. Ligands of GPCRs bind to its extracellular loops, N-terminal tail, or bindings sites in between its transmembrane helices, forming a cavity in its tertiary structure. GPCRs coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) are composed of three subunits: α, β, and γ. G proteins are classified based on the Gα subunit sequence homology into G_s, G_i, G_q, and G_{12} families. Ligand binding triggers a conformational change of the receptor, allowing coupling to the G protein. GPCR then acts as a guanine

nucleotide exchange factor and activates associated guanosine diphosphate (GDP) binding G protein by catalyzing the exchange of GDP for the guanosine triphosphate (GTP). GTP binding α subunit dissociates from the βγ subunit and triggers the intracellular signaling pathway.

7.1.1. Major Opioid Receptors

Following the discovery of opioid-like peptides, synthesized by neurons in various brain regions (Olson et al., 1979), techniques of gene cloning lead to the discovery of three endogenous opioid peptide systems encoded by genes for pre-proenkephalin, pre-proopiomelanocortin and pre-prodynorphin (Hughes et al., 1977 a; b; Zakarian et al., 1979; Goldstein et al., 1980; Horikawa et al., 1983). This finding in combination with the observation of naloxone-induced reversal of opioid analgesia in specific brain regions leads to a hypothesis that opioid drugs and endogenous opioid-like peptides produce analgesic effects by binding to specific receptors (Paterson et al., 1983). Cloning of distinct receptors with different selectivities for endogenous opioid-like peptides revealed three major opioid receptor subtypes – μ-opioid receptor (MOR), δ-opioid receptor (DOR), and κ-opioid receptor (KOR) (Kieffer et al., 1992; Chen et al., 1993 a; b). The fourth major opioid receptor, cloned by homology with the opioid receptors, is the most recently discovered opioid receptor, and was at first named opioid receptor like receptor 1 (ORL1) (Mollereau et al., 1994). Later discovery of neuropeptide nociceptin/orphanin FQ, an endogenous ligand of ORL1 (Meunier et al., 1995), led to a reevaluation of the nomenclature of the ORL1 and a change to nociceptin opioid peptide receptor (NOP) (Cox et al., 2015). Opioid receptors are GPCRs of class A (Rhodopsin) that couple to the $G_{i/o}$ family of G proteins, or pertussis toxin-sensitive guanine nucleotide-binding proteins. Opioid receptors share many structural similarities and intracellular signaling mechanisms; upon dissociation, $G_{i/o}$ proteins transduce opioid signaling primarily by inhibition of the cAMP-dependent pathway by inhibition of adenylyl cyclase activity, activation of G protein-coupled inwardly rectifying potassium channels (GIRKs) increasing potassium conductance, and inhibition of voltage-gated calcium channels.

The distribution of opioid receptors spans throughout the CNS and PNS, with particularly high density in various key regions along the ascending and descending pain pathways (Mansour et al., 1995). The most important site of action for opioids in analgesia is the dorsal horn of the spinal cord. Additional prominent sites of actions of opioids,

responsible for opioid-induced analgesia, are located in the brain stem - periaqueductal gray, n. raphe magnus, n. reticularis gigantocellularis, and locus coeruleus. Analgesia induced by topical administration of opioids lacking significant systemic absorption and a presence of opioid receptors on DRG and peripheral nerves provides evidence of a peripheral component of opioid-induced analgesia (Stein et al., 2003; Kolesnikov et al., 2004).

On a synaptic level, opioid receptors are found both on presynaptic and postsynaptic junctions, inducing both inhibitory and excitatory actions on neuronal synapses. Opioid receptors on the postsynaptic membranes elicit activation of GIRKs of a Kir channels family, consisting of Kir3.1, Kir3.2, Kir3.3, and Kir3.4 subunits, forming homo or heterotetrameric GIRK channels. The major subtype present in the CNS is GIRK2 (Kir3.2) and upon activation by opioids induces hyperpolarization of a neuron as the GIRK2 channel facilitates outward K^+ current, thus promoting inhibitory action of opioids.

An important presynaptic mechanism of opioid-induced inhibitory actions is through N-type voltage-gated calcium channels (VGCC). VGCCs are located on the presynaptic membranes of neurons in both PNS and CNS and facilitate calcium influx upon activation causing neurotransmitter release into the synaptic cleft (Mochida, 2018). Gβγ subunit of opioid receptors interact with N-type VGCCs and inhibit Ca2+ current through the channel, and in turn, block the release of neurotransmitters.

The first discovered and most extensively studied distinct opioid receptor subtypes are MORs. MOR is the dominant opioid receptor in pain-relieving effects as well as a receptor with the highest affinity for morphine, the most important and frequently used opioid in medicine. At the same time, MORs are responsible for most of the adverse side effects of opioids, such as tolerance, addiction, dependence, respiratory depression, and constipation. For this reason, modern research on opioid analgesia is focused on achieving opioid-induced analgesia through biased agonism of MOR or identifying safe-to-use, effective ligands of DOR and KOR, that can substitute nowadays widely used opioids targeting MOR.

7.1.2. Opioid-induced hyperalgesia

As described in previous chapters, hyperalgesia refers to an enhanced pain perception from a normally painful stimulus. Hyperalgesia is a common symptom in patients with

neuropathic pain but can paradoxically be a side effect of opioid analgesics. In general, prolonged exposure to opioids can lead to a reduction of opioid receptors on the cell membrane, leading to a decrease in susceptibility to opioids and heightened pain sensitivity, referred to as OIH (Colvin et al., 2019).

Recent studies indicate the involvement of TRPV1 in OIH (Chen et al., 2008; Scherer et al., 2017). In a rat model of opioid tolerance and associated OIH, induced by a chronic morphine treatment, TRPV1 expression increased in the sciatic nerve, DRG, and spinal cord (Chen et al., 2008). Morphine-induced opioid tolerance in mice can be attenuated by intraperitoneal (i.p.) treatment of TRPV1 antagonist capsazepine (Nguyen et al., 2010). Both thermal and tactile OIH induced by a repetitive morphine treatment in mice and rats can be reversed by oral administration of TRPV1 antagonist AMG0374, while subcutaneous morphine pellets failed to induce thermal and tactile OIH in TRPV1 knock-out mice (Vardanyan et al., 2009). As was demonstrated on peripheral MORs, activation of MOR by a highly selective agonist of MOR [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) induced recruitment of β-arrestin 2 to MOR, leading to sensitization of TRPV1 in nociceptive neurons (Rowan et al., 2014). Taken together, TRPV1 receptors seem to exert a high level of control over opioid-induced tolerance and hyperalgesia and can be targeted to combat these side effects of opioid treatment.

7.1.3. Mu-opioid receptor desensitization

Receptor desensitization is a critical process in the signaling regulation, essential for the control of cell physiology. Desensitization in essence prevents excessive activation of the receptor by the sustained presence of its agonist. As with other members of the GPCR receptor family, desensitization regulates MOR by reducing the response to re-stimulation and plays a role in the development of opioid tolerance (Corder et al., 2017). Homologous desensitization of MOR – desensitization induced by a homologous agonist - takes place within minutes after the activation of the receptor and can last for tens of minutes after the removal of the agonist that induced the desensitization (Williams, 2014). The desensitized receptor can remain on the plasma membrane in an unresponsive state or can be internalized into intracellular complexes. Pivotal mechanisms taking place in agonist-induced MOR desensitization involve receptor phosphorylation by GRKs and β-arrestin-induced internalization of the receptor.

MOR internalization is dependent on β-arrestins, which promote the G protein uncoupling and endocytosis of the receptor (Pierce et al., 2002). Different agonists trigger various desensitization pathways of MOR - DAMGO-induced activation leading to recruitment of β-arrestin 2 and 3, while binding of morphine can evoke arrestin-dependent internalization by β-arrestin 3 (Groer et al., 2011). β-arrestin signaling can be involved in the development of opioid tolerance, as a complete lack of tolerance was observed in β-arrestin 2 knock-out mice (Raehal et al., 2005). β-arrestin 2 recruitment is enabled by receptor phosphorylation via GRK and leads to receptor endocytosis (Smith et al., 2016). Thus β-arrestin 2 sits in the center of MOR function and desensitization regulation.

Recent research focused on TRPV1-MOR interaction, with β-arrestin 2 as a central hub in communication between these receptors. At first, TRPV1 agonist capsaicin attenuated opioid-induced MOR desensitization in human embryonic kidney cells, evaluated as the duration of MOR activity upon agonist binding. Later study reveals that activation of TRPV1 in cells expressing TRPV1, β-arrestin 2, and MOR, leads to acute trafficking of β-arrestin 2 to the nucleus, thereby restricting the availability of β-arrestin 2 in the cytosol (Basso et al., 2019). TRPV1-induced trafficking thus hinders the ability of β-arrestin 2 to mediate the desensitization of MOR. This finding suggests that TRPV1 can bias MOR signaling and maintain the sensitivity of MOR to both endogenous and exogenous agonists (Basso et al., 2019).

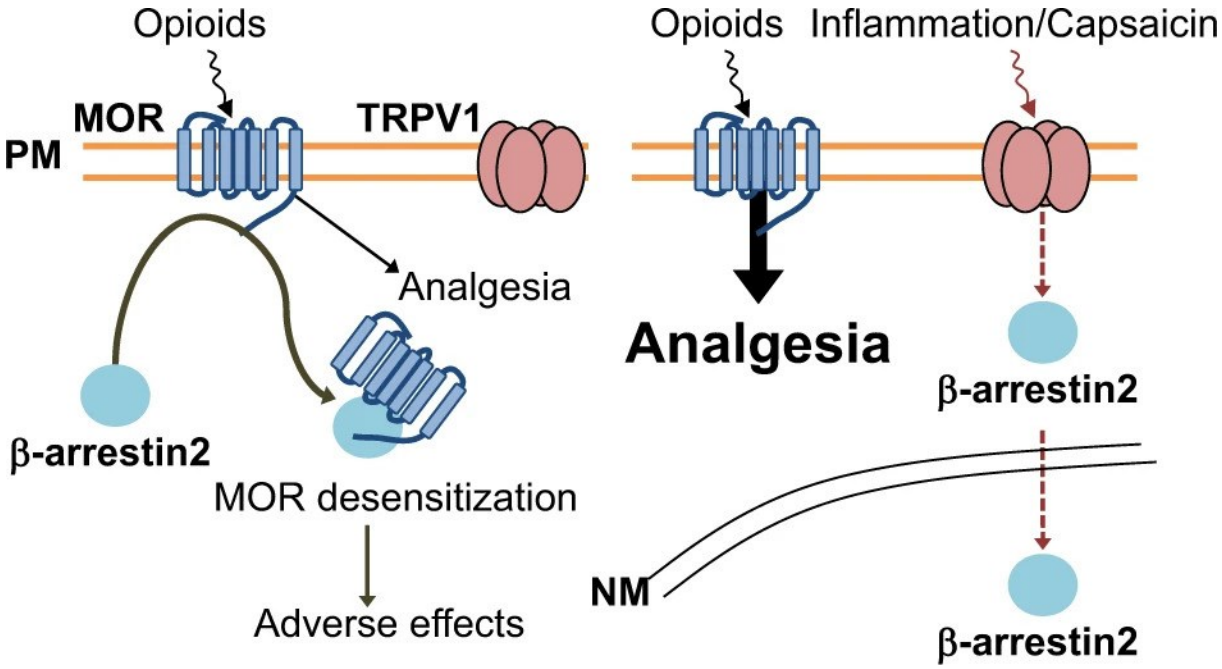

Figure 7.1.: **Schematic illustration of TRPV1-MOR interaction.** The left side of the image represents a situation where TRPV1 is inactive and activation of MOR by its agonist leads to β-arrestin 2 mediated MOR desensitization. Activation of the TRPV1 illustrated on the right shows how TRPV1 can prevent MOR desensitization through β-arrestin 2 translocation, potentially enhancing opioid-mediated analgesia. Adapted from (Abdullah et al., 2020).

The reduced analgesic potency of opioids is an important component of neuropathic pain and recent studies demonstrated the involvement of chemokines in opioid receptors desensitization. While opioids exert an analgesic effect in the CCI model of neuropathic pain, administration of morphine in combination with a CCR2 antagonist substantially enhanced the analgesic potency (Kwiatkowski et al., 2017). Another study demonstrated that the CCL2 neutralizing antibody attenuates morphine-induced microglial activation, hence effectively reducing the development of morphine tolerance (Zhao et al., 2012). Aside from the chemokine-induced microglial activation, direct inhibitory action of several chemokines through heterologous desensitization of MOR has been demonstrated (Zhang et al., 2004). Heterologous desensitization and subsequent internalization of MOR in cells expressing MOR and chemokine receptors were induced by CCL2, CCL3, CCL5, and CXCL8 and resulted in a marked inhibition of MOR function. This phenomenon might play an important role in inflammatory and neuropathic pain, where both peripheral and central neurons of nociceptive pathways are exposed to high levels of

proinflammatory cytokines. Chemokine-induced desensitization of MOR might compromise MOR function, attenuate analgesic effects of opoids and impair endogenous opoiod system. These findings reveal that chemokines promote development of chronic pain by a combination of intrinsic pronociceptive effects and a dysregulation of inhibitory mechanisms.

Part II.

Experimental work

8. Aims of the Ph.D. project

The aim of my Ph.D. project and the experiments presented in this thesis was to study various mechanisms implicated in the modulation of neuropathic pain. The main part of the project focused on the interaction between neuropathic pain-related chemokine CCL2, opioid receptors, and TRPV1 receptors in the spinal cord dorsal horn. The second part of the experiments studied the role of PI3K in PIPN.

Opioids under normal conditions exert potent inhibitory control over the nociceptive transmission. Neuropathic pain is associated with abnormal activity of nociceptive transmission in the SCDH and is often accompanied by attenuated opioid-mediated analgesia. TRPV1 receptors are involved in the pro-nociceptive effects of CCL2, and play a role in the trafficking of opioid receptors.

PAC application leads to PIPN, acute and chronic pain syndromes. While the role of DRG neurons in this pathological process was demonstrated, the molecular mechanisms are still unknown.

Based on these facts, we investigated the following experimental questions:

1. How chemokine CCL2 modulates MOR-mediated effects on nociceptive synaptic transmission in SCDH neurons and *in vivo* during opioid-mediated analgesia?

2. Are CCL2-mediated effects dependent on microglia activation?

3. What is the role of TRPV1 receptors in the development of OIH and how can the activation of TRPV1 by endogenous agonist OLDA modulate the opioid receptor desensitization?

4. Which intracellular signaling pathways are involved in PIPN and how can their inhibitors attenuate changes in DRG related to the development of neuropathic pain, such as macrophage infiltration?

9. Methods

9.1. Statement of Ethical Consideration

All experiments were approved by the Animal Care and Use Committee of the Institute of Physiology CAS and were in accordance with the guidelines of the International Association for the Study of Pain, EU Directive 2010/63/EU, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

9.2. Animals

Male P19–P21 rats were used for spinal cord slice electrophysiology and adult male Wistar rats were used for behavioral and immunohistochemical experiments. The animals were housed in separate clear plastic cages with soft bedding, free access to food and water, and maintained on 12 hours light/12 hours dark cycle at room temperature-controlled conditions. The experiments were carried out during the light phase of the cycle.

9.3. Spinal cord slice preparation

Animals were anesthetized with isoflurane (3%), and the lumbar spinal cord was removed and immersed in an oxygenated ice-cold dissection solution containing (in mM) 95 NaCl, 1.8 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, and 50 sucrose. Animals were sacrificed by subsequent medulla interruption and exsanguination. The spinal cord segment was fixed to a vibratome stage (VT 1000S, Leica, Germany) using cyanoacrylate glue in a groove between two agar blocks. Acute transverse slices 300–350 μm thick were cut from L4 to L5 segments, incubated in the dissection solution for 30 min at 33 °C, stored in a recording solution at room temperature, and allowed to recover for 1 h before the electrophysiological experiments. Recording solution contained (in mM) 127 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 25 D-glucose. Slices used in the experiments with minocycline were incubated with minocycline alone for 15 min before the addition of CCL2. In experiments with agonist-induced desensitization of opioid receptors, slices were incubated with DAMGO or with DAMGO + OLDA for 2 hours before the measurement. For the actual measurement, slices were transferred into a recording chamber that was perfused continuously with a

recording solution (room temperature) at a rate of ~ 2 ml.min^{-1}. All extracellular solutions were saturated with carbogen (95% O2, 5% CO2) during the whole process.

9.4. Patch-clamp recording

Patch-clamp recordings in spinal cord slices were made from superficial dorsal horn neurons in lamina I and II(outer). Individual neurons were visualized using a differential interference contrast (DIC) microscope (Leica, DM LFSA, Germany) equipped with a near-infrared-sensitive camera (Hitachi KP-200P, Japan) with a standard TV/video monitor. Patch pipettes were pulled from borosilicate glass tubing with resistances of 3.5–6.0 MΩ when filled with intracellular solution. The intracellular pipette solution contained (in mM): 125 gluconic acid lactone, 15 CsCl, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 MgATP, 0.5 NaGTP and was adjusted to pH 7.2 with CsOH. Voltage-clamp recordings in the whole-cell configuration were performed with an Axopatch 200B amplifier and Digidata 1440A digitizer (Molecular Devices, USA) at room temperature (~ 23 °C). Whole-cell recordings were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. The series resistance of neurons was routinely compensated by 80% and was monitored during the whole experiment. AMPA receptor-mediated miniature and evoked EPSCs were recorded from neurons clamped at − 70 mV in the presence of 10 μM bicuculline and 5 μM strychnine. Miniature EPSCs (mEPSC) were distinguished by the addition of 0.5 μM tetrodotoxin (TTX) to the bath solution. To record evoked EPSCs (eEPSC), a dorsal root was stimulated using a suction electrode with a glass pipette filled with an extracellular solution using a constant current isolated stimulator (Digitimer DS3, England). The intensity of the stimulation was adjusted to evoke stable EPSC with 0.5 ms stimulus duration and at least 3 × the minimal stimulus current at a frequency of 0.033 Hz.

9.5. Intrathecal catheter implantation

All i.t. drug deliveries in our experiments utilized i.t. catheters. Catheters were made of PE-5 and PE-10 tubing. Approximately 4 cm long PE-5 tube was inserted inside bent end of PE-10 tube and fixed using epoxy-glue. Tubing was filled with a sterile saline solution using a Hamilton syringe. Adult male Wistar rats (250–300 g) were anesthetized with isoflurane (3%), and the back of the animal was clipped with an electric razor. The surgery was performed in a sterile manner. After locating and marking the L4 and L5

vertebrae, a longitudinal incision through the skin and subcutaneous tissue above the spine was made to expose the lumbar vertebrae. Approximately 0,5 cm of PE-5 end of the catheter was placed into the subarachnoid space and fixed in place with dental cement (Duracryl, Spofa, Czech Republic). The adjacent surface was cleaned using fine forceps to allow for the proper bond of dental cement to vertebrae. The PE-10 end of the catheter was placed beneath the animal's skin and exposed on the back of the neck. The wound was surgically closed in layers and the open end of the catheter was sealed using heat-coagulation. All the animals were tested before the control experiments during the behavioral acclimatization period for any signs of any neurological deficits due to the catheter implantation. None of the animals used in the experiments showed any neurological deficits. Animals were left to recover in their cages for at least 7 days. The position of the catheters was verified by a dye injection at the end of each experiment and only data from animals with properly implanted catheters were used for the study.

9.6. Paw withdrawal latency to thermal stimulation

Adult male Wistar rats (250–300 g, n = 11) used in experiments were housed with a 12 h light/12 h dark cycle and in standard conditions with food and drinking water available ad libitum. The paw withdrawal latency (PWL) to thermal stimulation was tested using a plantar test apparatus (Ugo Basile, Italy) with radiant heat applied to the plantar surface of each hind paw. Rats were placed in nonbinding, clear plastic cages on a clear glass plate, with a heat source underneath. Rats were left to adapt to the testing environment for at least 15 min prior to any stimulation. Each paw was tested 4 times at each time point of the experiment with at least 5 min between the trials. Control values of PWL were acquired at the beginning of each experimental protocol before drug application. During the initial experiment, a single DAMGO administration was given, and the responses were evaluated. Two days later, the same animals received a single i.t. application of CCL2 followed by DAMGO 30 min later. PWL to thermal stimuli was assessed before CCL2 (control value) and after DAMGO application (at 30 min, 1, 2, and 4 h after injection). The averaged values from the hind paws of individual animals were averaged in the experimental groups. In the experiments exploring the interaction between opioids and TRPV1, animals received 0,01 μg of DAMGO and a combination of 0,42 μg of OLDA and 1,4 μg of CCL2 followed by 0,01 μg of DAMGO 30 minutes later. Control values of PWL were acquired at the beginning of each experimental protocol before drug

application. Data from experiments with plantar test apparatus were analyzed by One Way ANOVA followed by multiple comparison procedure versus control (Bonferroni post hoc test). The criterion for statistical significance was a $P < 0.05$.

9.7. Paclitaxel CIPN model, Duvelisib and Wortmannin treatment

PAC Mylan (Oncotec Pharma Produktion) was used to induce painful peripheral neuropathy in rats. An original clinically used stock solution of PAC (6 mg/ml) was diluted with 0.9% sterile saline to 2 mg/ml, just before injection. To replicate the original PAC formulation, a vehicle stock solution was made using 1:1 macrogolglycerol ricinoleate (Kolliphor EL, Sigma-Aldrich) and ethanol (Penta). Three successive doses of 8 mg/kg PAC were intraperitoneally administered in rats on days 0, 3, and 6, representing a final cumulative dose of 24 mg/kg. This dosage produces a preclinical model of chemotherapy-induced painful peripheral neuropathy in rats, as previously described (Liu et al., 2010; Li et al., 2015). In CTRL groups, animals were treated with a vehicle: the equivalent volume of Kolliphor EL and ethanol (1:1) in sterile saline. Pretreatment with wortmannin (0.6 mg/kg, i.p.) had preceded 1 h before the PAC treatment. The stock solution of wortmannin (12.5 mM in dimethyl sulfoxide (DMSO)) was diluted in sterile saline. The PAC-only treated animals in experiments with wortmannin received a sham injection (DMSO as a vehicle) 1 h before the PAC, corresponding to the wortmannin treatment. Duvelisib (DUV) (LC Laboratories) stock solution (up to 200 mM) was prepared freshly in DMSO (Sigma-Aldrich) just before the treatment. An appropriate dose of DUV was mixed with the vehicle (edible sunflower oil; 500 µl per rat) and administered via oral gavage into the stomach. Plastic feeding tubes (Instech Laboratories) 15 G x 78 mm were used. DUV (10 mg/kg) was administered as a pretreatment 30 min before the PAC.

9.8. Immunohistochemical analysis of Akt kinase phosphorylation

Wistar rats were randomly distributed in three experimental groups: Control (CTRL group, n = 5); paclitaxel (PAC group, n = 5); wortmannin + paclitaxel (WMN + PAC group, n = 5). Control animals received an equivalent volume of the vehicle (1:1 Kolliphor EL: ethanol) diluted in saline, the PAC group received 8 mg/kg i.p. Paclitaxel (Mylan) and animals in the WMN + PAC group were pretreated with wortmannin (0.6

mg/ kg) 1 h before PAC administration. Animals were 1 h later deeply anesthetized with a combination of ketamine (100 mg/kg, Narketan, Zentiva) and xylazine (25 mg/kg, Xylapan, Zentiva), perfused intracardially with saline followed by ice-cold 4% paraformaldehyde. Both L5 DRGs were removed and post-fixed in 4% paraformaldehyde at 4° C for 2 h, cryoprotected with 30% sucrose overnight, and cut in cryostat Leica CM3000 to 16 μm thick slices. Every 3rd DRG section was then processed for phosphorylated Akt kinase (pAkt) immunohistochemistry. Briefly, sections were washed 3× for 10 min in phosphate-buffer solution (PBS), blocked with 3% normal donkey serum (NDS) for 30 min at room temperature, and incubated overnight at 4° C with rabbit anti-pAkt (Ser473) (1:200; Cell Signaling Technology, #4060S, Netherlands) primary antibody in 1% NDS with 0.3% Triton X-100. After washing in 1% NDS (3× for 10 min), the sections were exposed to a donkey anti-rabbit Cy2-conjugated secondary antibody (1:400, Jackson ImmunoResearch Lab. Inc, USA) for 2 h. For visualization of the cell nucleus, incubation in bisbenzimide (Hoechst 33342, Sigma-Aldrich) for 3 min was used. Pictures from all sections were captured using a digital camera on a fluorescence microscope (Olympus BX53) and analyzed offline using ImageJ software (NIH, USA) by an investigator blinded to the treatment. Region of interest (ROI) containing only neuronal cell bodies (excluding nerve/root fibers) was outlined for each DRG section and the immunoreactive (IR) area of pAkt was done in ROI using the Threshold function. To set the proper Threshold value, we obtained intensity values of multiple pAkt IR cell bodies first. A minimum of 5 sections were evaluated in each ganglion. IR/ROI ratios were calculated and expressed as a percentage. To analyze the number of pAkt IR DRG cells, all cell bodies in the DRG section were manually outlined and the pAkt IR intensity and area of individual cell bodies were measured. Cell bodies were divided into pAkt IR and pAkt nonIR groups based on the signal intensity. Only cells with visible nucleus were included in the analysis (Control: n = 1000, PAC group: n = 838, WMN + PAC group: n = 1080).

9.9. Immunohistochemical analysis of CD68-immunoreactive macrophages in DRG

Adult Wistar rats were randomly distributed in three experimental groups: CTRL group (n = 6), PAC (n = 8), DUV + PAC group (n = 9). Animals received the standard vehicle, PAC, or DUV + PAC treatment on days 0, 3, and 6, as described above. PAC was

administered 3x8 mg/kg (i.p.) and DUV 10 mg/kg (p.o.) as 30 min pretreatment. Animals were perfused, and tissue was collected and processed (as described above) 24 h after the last treatment. Mouse anti-CD68 primary antibody [ED1] (1:100, Abcam, #ab31630) and a donkey anti-mouse AlexaFluor-488 secondary antibody (1:400, Jackson ImmunoResearch Laboratories, #715-545-151) were used. Slices were mounted by DPX mounting medium. Pictures were captured using a confocal microscope (Leica Microsystems, SP8). Multi-immersion objective HC PL APO (20x/0.75 NA), Ar multiline laser (488 nm; 65 mW), and HyD spectral detectors were used. Data were analyzed offline using ImageJ software (National Institutes of Health) by an investigator blinded to the treatment, using ROI containing only neuronal cell bodies area (excluding nerve/root fibers). CD68 IR area was analyzed in ROI using the Threshold function. Left and right L5 DRGs were analyzed separately (CTRL: n = 12, PAC group: n = 16, DUV + PAC group: n = 18).

10. Results

10.1. Chemokine CCL2 prevents opioid-induced inhibition of nociceptive synaptic transmission in spinal cord dorsal horn

10.1.1. DAMGO-induced depression of mEPSC frequency was attenuated by CCL2

Patch-clamp recordings in this study were obtained from nociceptive neurons predominantly localized in lamina I and II(outer). Verification of the nociceptive input of the neurons recorded from the dorsal horn was done by the capsaicin (0.1 μM) application at the end of the experimental protocol; only neurons with an increase of mEPSC or sEPSC frequency in the reaction to the capsaicin were included in the study.Short application of highly selective MOR agonist DAMGO (1 μM, 3 min) on acute spinal cord slices reduced mEPSC frequency to 60.8 ± 4.0% from the basal value at the 2nd and 3rd minutes of application ($P < 0.001$, $n = 16$, Fig. 10.1.A,E). DAMGO-induced inhibition of the mEPSC frequency reached its peak during the wash-out period, specifically 5 min after the cessation of DAMGO application (56.7 ± 5.9%, $P < 0.001$). In the case of the mEPSC recordings, DAMGO-induced frequency inhibition remained significant until the end of the recording (63.6 ± 6.4%, $P < 0.001$). An increase of the mEPSC frequency during the washout part of the recording was observed only in 1 of the recorded neurons (566%) and since the nociceptive input of the neuron could not be verified as it did not respond to capsaicin by an increase of mEPSC frequency, this neuron was excluded from the analysis. The mean amplitude of the mEPSCs was 19.3 ± 1.7 pA during the control part of the recording and we did not observe a significant DAMGO-induced change during application (95.2 ± 3.7% of the control, Fig. 10.1.F) or the washout period (5 min: 91.2 ± 4.6%; 10 min: 94.7 ± 5.8%; 15 min: 82.8 ± 5.4%).

We have tested the contribution of the TRPV1 receptor to the DAMGO-induced inhibition of mEPSC frequency using a specific TRPV1 antagonist SB366791. The application of SB366791 (10 μM) was applied on acute spinal cord slices throughout the whole recording protocol, starting 4 min before the DAMGO (1 μM, 3 min). SB366791 alone did not in any way affect the frequency of the mEPSCs (90.9 ± 7.4%, $n = 8$, Fig. 10.1.B). DAMGO-induced inhibition of mEPSC frequency in the presence of SB366791

was similar to the inhibition in experiments without SB366791; DAMGO-mediated mEPSC frequency depression peaked in the first minutes of the application (54.4 ± 4.7%, $P < 0.001$, Fig. 10.1.B, E) and remained significant throughout whole 15 min long washout period (15 min: 68.2 ± 8.0%, $P < 0.001$). DAMGO in combination with the SB366791 pre-treatment did not change the mEPSC amplitude (Ctrl: 16.5 ± 1.5 pA; SB: 91.3 ± 4.5%; DAMGO: 97.2 ± 6.0%; wash 5 min: 102.6 ± 8.4%; 10 min: 100.1 ± 6.0%; 15 min: 96.6 ± 6.9%).

After establishing the effect of DAMGO on mEPSC frequency in naïve slices, we aimed to study how elevated levels of CCL2 can change the effect of DAMGO. In the following experiments, spinal cord slices were pre-incubated with CCL2 (10 nM, 2 h) before recording. After exposure to CCL2, DAMGO depressed the mEPSC frequency only to 82.4 ± 3.9% ($P < 0.05$, $n = 11$, Fig. 10.1.C, E) from the basal value. The frequency of mEPSC remained approximately at the same level in the subsequent 15 min of the washout period. CCL2 pre-treatment and the application of DAMGO did not change the amplitude of the mEPSC (Ctrl: 16.8 ± 1.2 pA; DAMGO: 96.7 ± 5.5%, wash 5 min: 91.1 ± 5.6%; 10 min: 90.9 ± 5.0%; 15 min: 89.2 ± 6.6%, Fig. 10.1.F). This indicates that CCL2 pre-treatment robustly attenuated the inhibitory effect of the DAMGO application on the mEPSC frequency in the superficial dorsal horn neurons.

Although the contribution of microglia to the development of neuropathic and neuroinflammatory pain is well established, initial reports of microglia expressing CCR2, based on immunohistochemistry (Abbadie et al., 2003), had been disputed in recent studies utilizing CCR2 reporter mice (Pottorf et al., 2022). Despite the uncertainties that remain to be answered, most recent research suggests a CCR2-dependent mechanism of microglial activation (Pottorf et al., 2022). To study the contribution of microglia to the CCL2-induced inhibition of MOR signaling in SCDH neurons, spinal cord slices were pre-incubated with CCL2 and minocycline, a blocker of microglia activation, for 2 h before the recording. The addition of minocycline (100 μM) 15 min before the CCL2 (10 nM) did not have any influence on the suppressive effect of CCL2 on the DAMGO-induced inhibition. Acute application of DAMGO elicited the mEPSCs frequency depression to 77.4 ± 4.1% ($P < 0.05$, $n = 9$, Fig. 10.1.D, E). DAMGO-induced depression lasted throughout the washout period as in the CCL2 treatment group. The amplitude of the mEPSCs did not change during the recording (DAMGO: 81.0 ± 5.0%; wash 5 min: 80.5 ± 7.0%; 10 min: 84.5 ± 8.9%; 15 min: 89.7 ± 10.7%, Fig. 10.1.F). Capsaicin

application at the end of each mEPSC recording obtained in this study was used to confirm the nociceptive input of the recorded neuron; capsaicin induced a robust increase in the mEPSC frequency (2567 ± 727%, n = 44). Our results suggest that microglial activation is not an essential mechanism in the CCL2-mediated attenuation of the DAMGO-induced mEPSCs frequency decrease.

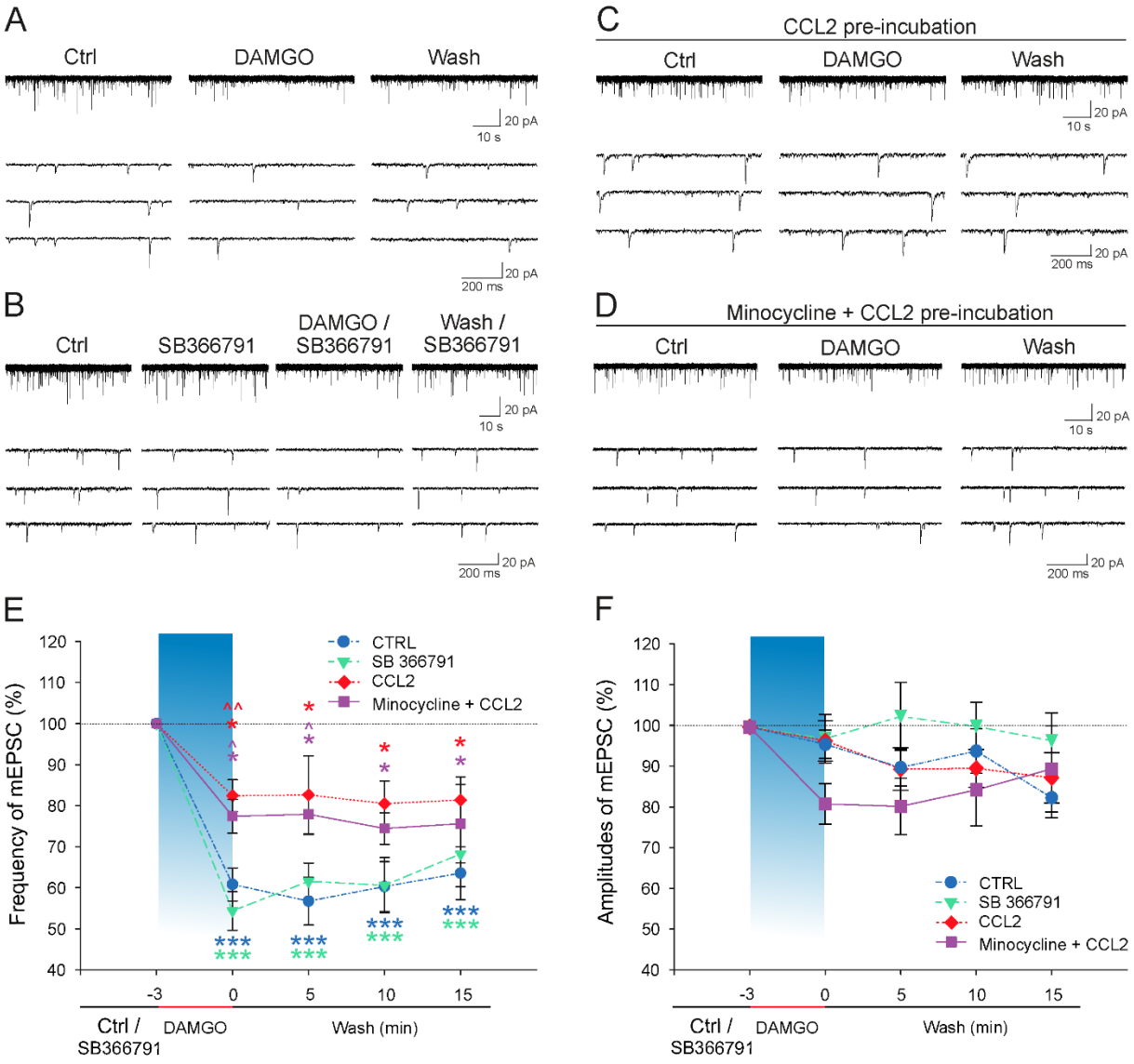

Figure 10.1.: **DAMGO-induced depression of mEPSC frequency was diminished by the CCL2 pre-treatment.** (**A**, **E**) Acute DAMGO (1 µM, 3 min, n = 16) depressed an mEPSC frequency; inhibition lasted throughout the 15 min washout period. (**B**, **E**) Specific TRPV1 antagonist SB366791 (10 µM, 4 min, n = 8) did not have any significant effect on the DAMGO-induced depression of the mEPSC

frequency. (**C**, **E**) Incubation of spinal cord slices with CCL2 (10 nM, 2 h) significantly attenuated DAMGO-induced inhibitory effect (n = 11). (**D**, **E**) Microglial activation blocker minocycline (100 μM) did not interfere with the CCL2-mediated attenuation of the DAMGO-induced inhibition of the mEPSC frequency (10 nM, 2 h, n = 9). (**F**) Mean mEPSC amplitudes did not significantly differ between the experimental groups, nor were affected by the DAMGO application. Statistical differences in each experimental group were identified using RM ANOVA on ranks followed by the Dunnett`s test; $^{*}P < 0.05$, $^{***}P < 0.001$ versus Ctrl/SB366791 part of the recording. Differences between the treatments were analyzed using one-way ANOVA followed by the Bonferroni test; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ versus CTRL group.

10.1.2. DAMGO-induced effects on eEPSC amplitude were modulated by TRPV1 inhibition and by CCL2 application via microglia activation

Following experiments aimed to study the effects of DAMGO and CCL2 on the nociceptive transmission using dorsal root electrical stimulation-evoked EPSCs. Recordings of eEPSC provide a closer reflection of the synaptic transmission properties than the mEPSCs. All of the 15 neurons recorded in the control group displayed a significant eEPSC amplitude decrease induced by the DAMGO (1 μM, 3 min) application, but amplitude changes during the washout period showed a distinctive difference between recorded neurons. 9 out of 15 neurons reacted to the DAMGO application by a rapid decrease of the mean eEPSC amplitude (43.8 ± 7.4%, n = 9, P < 0.05, Fig. 10.2.A, E) followed by a return to the pre-application amplitude (99.8 ± 16.0%) in the 7th min of the washout period. The eEPSC amplitude continuously increased and reached 166.6 ± 21.5% (P < 0.01) in the 17th min of the washout period (Fig. 10.2.E). The rest of the control group (6 out of the 15 recorded neurons) responded to the DAMGO application by a robust acute decrease of the eEPSC amplitude (33.0 ± 5.3%, n = 6, P < 0.001, Fig. 10.2.E). The amplitude depression remained significantly different from the control amplitude up to the 7th min of the washout (59.9 ± 10.2%, P < 0.01) and remained close to the basal value for the rest of the washout.

We applied SB366791 (10 μM, 4 min) as a pre-treatment before DAMGO and continuously for the rest of the recording to test the effect of TRPV1 receptor inhibition on the DAMGO-induced eEPSC amplitude modulation. Pre-treatment with SB366791 did not significantly change the amplitude of the eEPSCs compared to control

(90.0 ± 9.5%, n = 9). Following DAMGO/SB366791 application elicited a rapid decrease of the eEPSC amplitude (39.0 ± 9.8%, P<0.001, Fig. 10.2.B, E). Depression of the eEPSCs amplitude peaked at the 1st min of the washout period (34.3 ± 10.2%, P < 0.001, Fig. 10.2.E) and remained significantly inhibited in comparison to the control amplitude up to the 9th min of the washout (68.6 ± 10.3%, P < 0.05). The amplitude of the eEPSC continually increased and eventually reached the control value in the 18th min of the washout (98.8 ± 16.3%, Fig. 10.2.E). In contrast to the neurons recorded without SB366791, the delayed potentiation of the eEPSC amplitude was not present in any of the recorded neurons. Thus, inhibition of the TRPV1 receptors prevented the DAMGO-induced delayed increase of the eEPSCs amplitude.

To study how CCL2 affects DAMGO-mediated depression of eEPSCs recorded from the SCDH neurons, we pre-incubated acute slices with the CCL2 (10 nM, 2 h). Acute application of DAMGO (1 μM, 3 min) failed to induce a decrease of the eEPSCs amplitude in slices pre-incubated with the CCL2 (97.4 ± 10.7%, n = 11, Fig. 10.2.C, F). The eEPSC amplitude remained unchanged throughout the whole recording. Hence the incubation with CCL2 prevented not only the acute DAMGO-induced depression but also the TRPV1-dependent delayed eEPSC amplitude potentiation observed in the subpopulation of the neurons from the control group.

We tested the contribution of microglia activation using a protocol similar to the mEPSCs experiments. We pre-incubated acute spinal cord slices with minocycline (100 μM) and CCL2 (10 nM, 2 h). The minocycline was added to the incubation chamber 15 min before the CCL2. Acute application of DAMGO elicited significant depression of the eEPSC amplitude (56.4 ± 8.4%, n = 8, P < 0.001, Fig. 10.2.D, F). Amplitude throughout the washout did not exceed the control amplitude; DAMGO-induced delayed potentiation was not observed in any of the recorded neurons (Fig. 10.2.D, F). Mean control eEPSC amplitudes did not significantly differ between the control, SB366791, CCL2 and minocycline + CCL2 experimental groups (CTRL: − 433.9 ± 163.1 pA; SB366791: − 497.1 ± 123.6 pA; CCL2: − 392.3 ± 133.7 pA; minocycline + CCL2: − 744.5 ± 206.4 pA). Our results demonstrate how blocking the activation of microglia through minocycline treatment significantly attenuated the inhibitory effect of CCL2 on the DAMGO-induced eEPSC amplitude depression, while the increase of the eEPSCs amplitude during the washout period was absent.

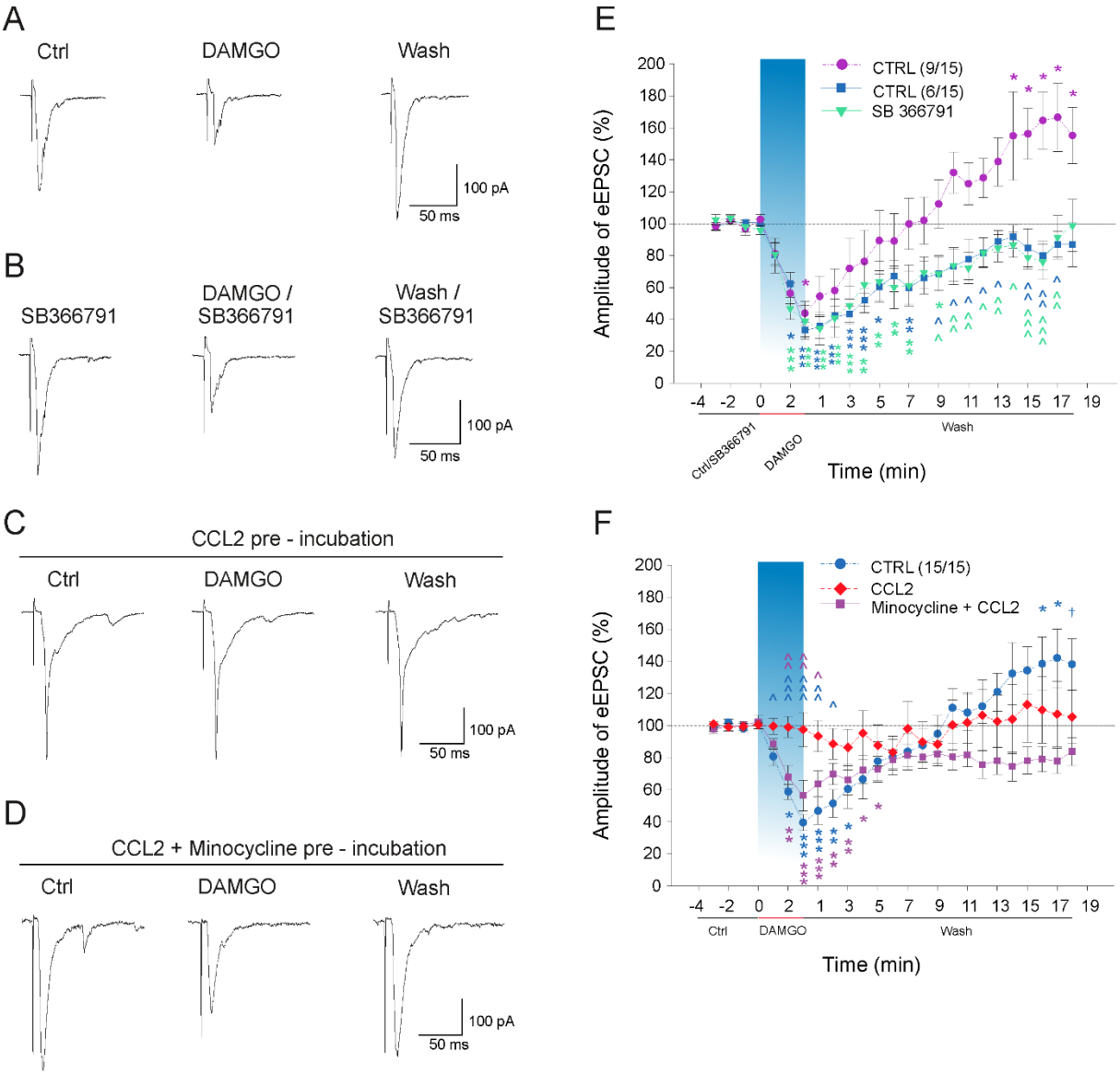

Figure 10.2.: **DAMGO-induced depression and delayed potentiation of the eEPSC amplitude is modulated by the TRPV1 antagonist and CCL2.** (**A**, **E**) Application of DAMGO (1 μM, 3 min, n = 15) depressed the eEPSC amplitude in all of the recorded SCDH neurons. DAMGO-induced depression was followed by a gradual increase of eEPSC amplitude during the washout in 9 out of the 15 neurons. (**B**, **E**) TRPV1 antagonist SB366791 (10 μM, 4 min, n = 9) did not affect the DAMGO-induced depression of eEPSC amplitude and prevented the delayed increase of eEPSC amplitude present in washout of the control group. (**C**, **F**) DAMGO failed to induce both eEPSC amplitude depression and delayed potentiation in neurons from slices pre-incubated with CCL2 (10 nM, 2 h, n = 11). (**D**, **F**) Microglia activation blocker minocycline

(100 μM, $n = 8$) prevented the inhibitory effect of CCL2; DAMGO elicited a significant depression of the eEPSC amplitude. Statistical differences in each experimental group were identified using RM ANOVA followed by the Bonferroni test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Ctrl/SB366791 part of the recording. Differences between treatments were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ versus CTRL (**E**) or CCL2 group (**F**); $^{+}P < 0.05$ versus minocycline group

10.1.3. CCL2 attenuates DAMGO-induced hyposensitivity to thermal stimuli in vivo

We used behavioral experiments to study the impairment of DAMGO-induced inhibition of nociceptive transmission, observed in previously described *in vitro* experiments. This experimental approach allowed us to see how changes in SCDH neuronal activity translate into modulation of DAMGO-induced analgesia (Fig. 10.3.). An i.t. catheter was used for the delivery of DAMGO (0.01 μg) and CCL2 (1.4 μg) to the spinal cord. Acute i.t. DAMGO injection significantly prolonged PWL to thermal stimuli. Analgesic effect of DAMGO peaked at 30 min after the application (196.9 ± 12.5% of the control PWL, n = 5, P < 0.01; Fig. 10.3.). Thermal hyposensitivity returned to normal gradually in course of the experiment, reaching 154.2 ± 16.2% 1 h after the DAMGO application and diminished 2 h later (120.2 ± 7.3%). CCL2 + DAMGO experimental group received CCL2 first, followed by DAMGO. CCL2 (1.4 μg) was delivered as a pre-treatment 30 min before the DAMGO (0.01 μg). DAMGO after CCL2 failed to significantly prolong PWL to the thermal stimuli in any of the assessed time points (30 min: 121.7 ± 10.2%; 1 h: 97.7 ± 11%; 2 h: 94 ± 5.2%; 4 h: 107.2 ± 1.7%; n = 5). Pre-treatment with CCL2 was able to completely diminish the analgesic effect mediated by spinal MOR activation.

Microglial blocker minocycline in a single i.p. dose (45 mg/kg) administered 1h before the CCL2 was used to study the possible role of the microglial activation on the CCL2-induced attenuation of the DAMGO-mediated analgesic effect. DAMGO application after minocycline and CCL2 pre-treatment was able to significantly prolong the PWL to thermal stimuli in all of the tested animals up to 1 h after the DAMGO (30 min: 200.5 ± 9.5%, P < 0.001; 1 h: 169.2 ± 7.6%, P < 0.01; 2 h: 140.8 ± 5.9%; 4 h: 115.1 ± 5.5%, n = 6). This result demonstrates how blocking the microglial activation can prevent the CCL2-induced attenuation of MOR-mediated thermal analgesia.

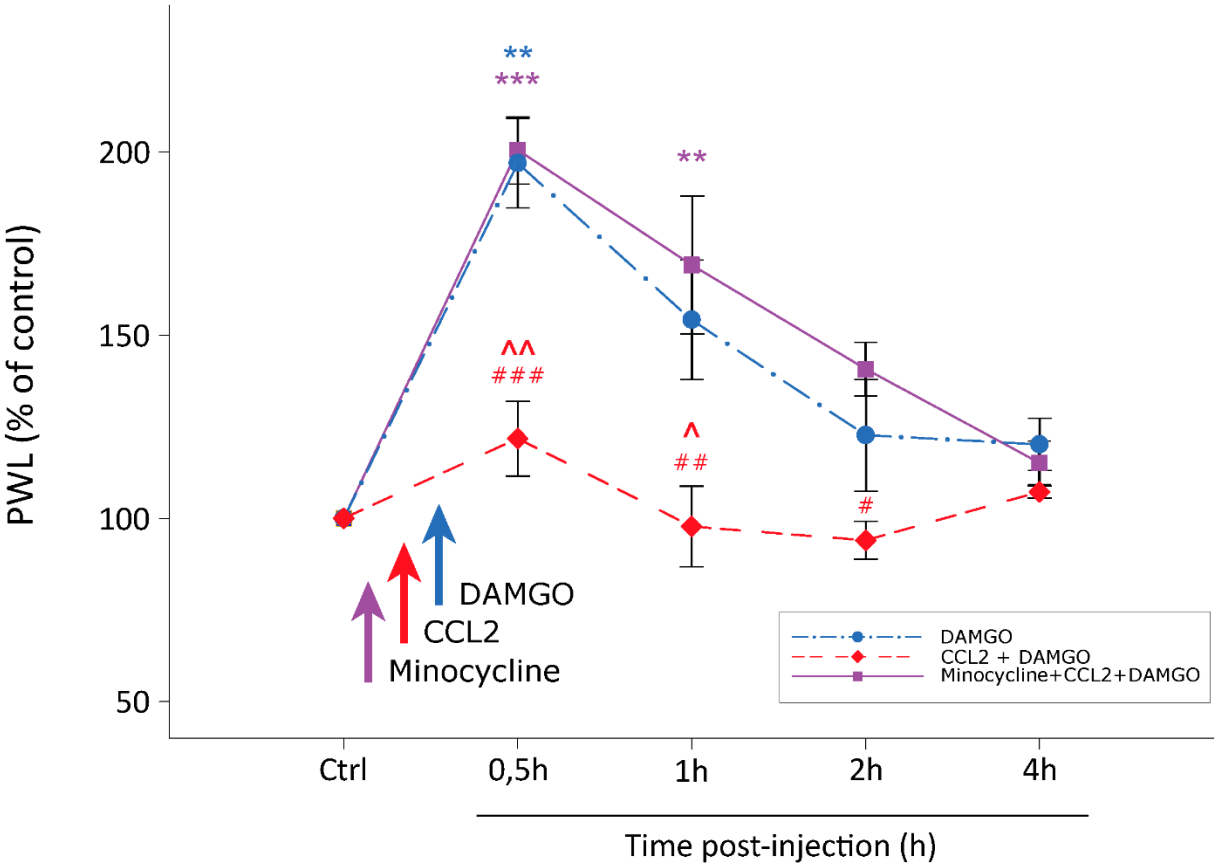

Figure 10.3.: **MOR-mediated thermal hyposensitivity induced by i.t. application of DAMGO was attenuated by the CCL2 pre-treatment in a microglia activation-dependent manner.** Acute i.t. application of DAMGO (0.01 µg, n = 5) elicited an increase of the PWL to thermal stimuli, lasting for up to 1 h after DAMGO. A single i.t. CCL2 (1.4 µg, n = 5) pre-treatment 30 min before the DAMGO (0.01 µg) prevented the MOR-mediated thermal analgesia. PWL remained unaffected throughout the testing period. Single i.p. application of minocycline (45 mg/kg, n = 6) eliminated the effect CCL2 had on the DAMGO-evoked thermal analgesia in all of the tested animals; the increase of PWL remained significant for up to 1 h after the DAMGO. Statistical differences in each experimental group were identified using RM ANOVA on ranks followed by Dunnett`s test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Ctrl. Differences between treatments were analyzed using one-way ANOVA followed by the Bonferroni test. $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ versus DAMGO group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Minocycline + CCL2 + DAMGO group

10.2. Endogenous TRPV1 agonist prevents MOR desensitization

10.2.1 OLDA prevented DAMGO-induced desensitization of MOR in the spinal cord

All of the mEPSC recordings were recorded from a population of nociceptive neurons predominantly localized in lamina I and II(outer) of acute spinal cord slices. The nociceptive input of the recorded neurons was verified by an increase in mEPSC frequency after the application of capsaicin (0.1 μM) at the end of the experimental protocol.

Agonist-induced desensitization of MOR was studied using acute spinal cord slices pre-incubated with 1 μM DAMGO for 2 h before the recording. Short application of DAMGO (1 μM, 3 min) on naïve acute spinal cord slices reduced mEPSC frequency to 60.8 ± 4.0% at the 2nd and 3rd minutes of the application ($P < 0.001$, n = 16, Fig. 10.4.A, B, D) and to 56.7 ± 5.9% and 63.6 ± 6.4% 5 and 10 min after the application, respectively ($P < 0.001$, Fig. 10.4.B, D). However, acute application of DAMGO failed to depress the mEPSC frequency in neurons recorded from the slices pre-incubated with DAMGO (1 μM, 2 h; 91.27 ± 4.4%, n = 12, Fig. 10.4.A, B, D). The complete lack of typical response to selective MOR agonist DAMGO in our recordings indicates functional desensitization of MOR, induced by prolonged agonist activation.

Recent studies suggest a close interaction between MOR and TRPV1, describing priming of MOR by the activation of TRPV1 and even prolonged opioid-induced signaling, which can be achieved in cell cultures by simultaneous activation of MOR and TRPV1 (Scherer et al., 2017; Basso et al., 2019). Moreover, data obtained from both cell cultures expressing TRPV1 and MOR and from primary trigeminal ganglion neuronal cultures provide evidence of bi-directional crosstalk between TRPV1 and MOR through the β-arrestin 2, implicated in MOR internalization (Groer et al., 2011; Rowan et al., 2014; Melkes et al., 2020). Because all of the studies mentioned above focused on either capsaicin (Rowan et al., 2014; Scherer et al., 2017; Melkes et al., 2020), or inflammation-induced activation of TRPV1 (Basso et al., 2019), there are no available studies describing the role of endogenous TRPV1 agonists in TRPV1-MOR crosstalk. To study the role of endogenous agonist-induced TRPV1 activation in agonist-induced MOR desensitization, we co-incubated the acute spinal cord slices with DAMGO and endogenous TRPV1 agonist OLDA in two concentrations (0.2 and 10 μM), based on our

previous studies (Spicarova et al., 2009, 2010). Both concentrations of OLDA were successful in preventing the DAMGO-induced desensitization of MOR. Short application of DAMGO (1 μM, 3 min) depressed the mEPSC frequency in slices co-incubated with the 10 μM OLDA + 1 μM DAMGO to 55.48 ± 6.07% ($P < 0.01$, $n = 6$, Fig. 10.4.A,C,D). The DAMGO-induced mEPSC frequency depression lasted for 10 minutes after the cessation of DAMGO (5 min: 62 ± 7.06%, $P < 0.01$; 10 min: 65.39 ± 7.62%, $P < 0.05$, 10.4.C,D). A lower concentration of OLDA (0.2 μM) showed a similar effect on DAMGO-induced MOR desensitization and preserved the function of MOR in the prolonged presence of DAMGO. Short application of DAMGO (1 μM, 3 min) on slices pre-incubated with 0.2 μM OLDA + 1 μM DAMGO depressed the frequency of mEPSC to 63.44 ± 7.65 % ($P < 0.01$, $n = 12$, 10.4.A,C,D). Significant DAMGO-induced depression in slices incubated with 0.2 μM OLDA was evident only during the DAMGO application and frequency quickly returned to control levels, showing that OLDA prevents agonist-induced MOR desensitization in a concentration-dependent manner.

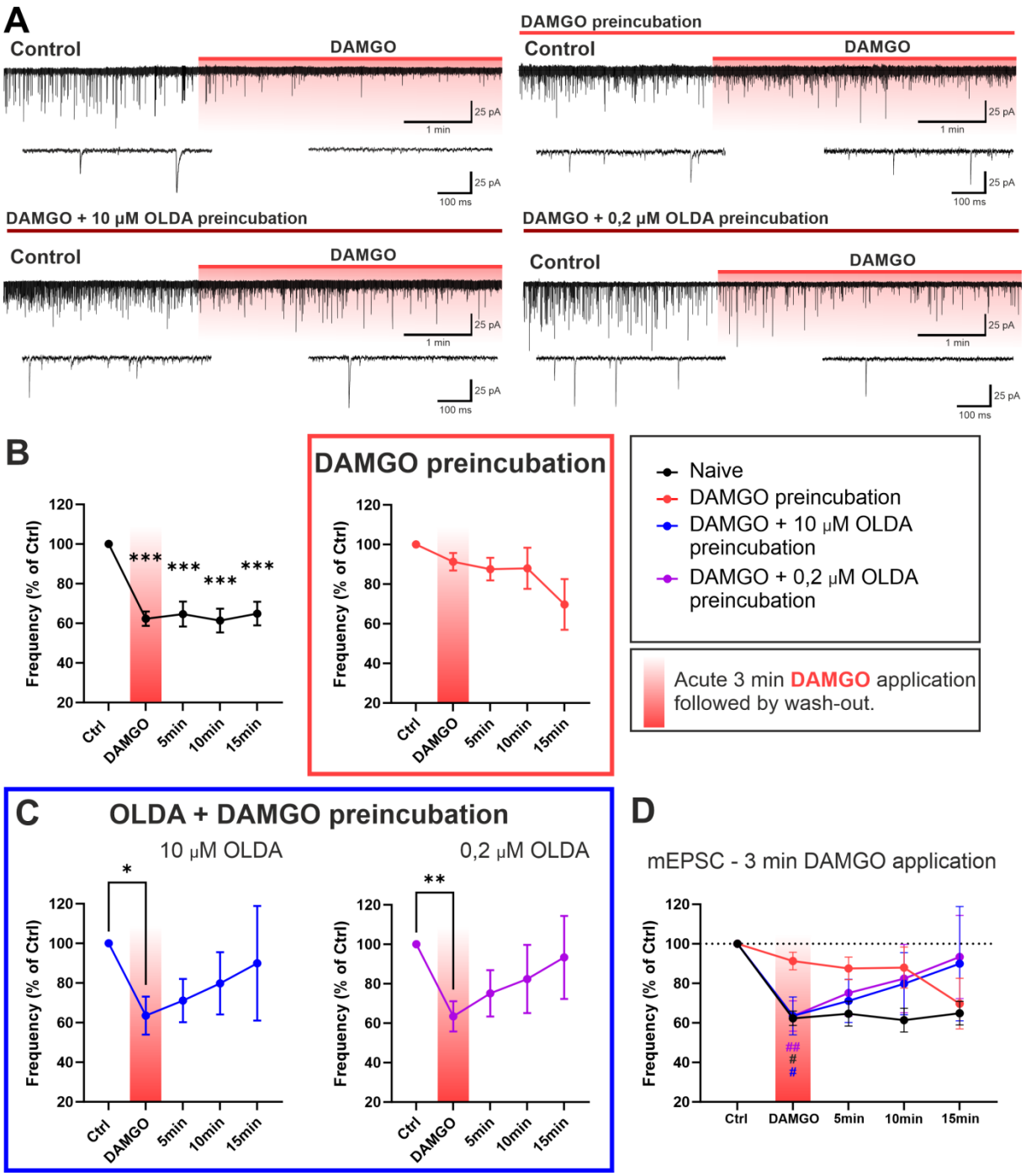

Figure 10.4.: **OLDA prevented DAMGO-induced desensitization of MOR in acute spinal cord slices from naïve rats.** (**A**) Illustrative mEPSC recordings demonstrating the effect of application of MOR agonist DAMGO (**B**) Short application of MOR agonist DAMGO (1 µM, 3 min) depressed mEPSC frequency in naïve slices. To induce robust MOR desensitization, we incubated slices with 1 µM DAMGO for 2 hours; acute application of DAMGO after the incubation failed to depress the mEPSC frequency. (**C**) The addition of endogenous TRPV1 agonist OLDA to DAMGO during incubation prevented the MOR desensitization in a concentration-dependent manner. Statistical

differences in each experimental group were identified using RM ANOVA on ranks followed by Dunnett`s test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Ctrl. (**D**) Summary of mEPSC frequencies from all of the recorded groups. Differences between the treatments were analyzed using one-way ANOVA followed by the Bonferroni test; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus CTRL group.

10.2.2. OLDA prevented CCL2-induced inhibition of MOR-mediated analgesia

We have used the plantar test apparatus to test the PWL to thermal stimulation and i.t. catheter administration to confirm that OLDA-mediated TRPV1 activation can prevent MOR desensitization *in vivo*. To induce MOR desensitization, we have repeated the previously published experimental protocol of i.t. CCL2-induced inhibition of MOR-mediated thermal analgesia (Heles et al., 2021). Rats in the CCL2 group received a single i.t. injection of CCL2 (1.4 μg, n = 5) followed by a single application of DAMGO (0.01 μg) 30 min after CCL2. Similarly to our previously published results, CCL2 prevented DAMGO-induced thermal hyposensitivity in the thermal plantar test (30 min: 123.3 ± 11.3%, P = 0.265, Fig. 10.5.). To test the effect of TRPV1 activation, we co-administered CCL2 (1.4 μg, n = 5) with OLDA (0.42 μg) 30 min before DAMGO (0.01 μg). DAMGO administered after CCL2 + OLDA induced rapid and potent hypoalgesia to thermal stimulation, measured as an increase in PWL, reaching 175.4 ± 18.7 % (P < 0.05) 30 min after DAMGO and 146.9 ± 4 % (P < 0.01) 1 h after DAMGO (Fig. 10.5.). Thus, activation of TRPV1 by OLDA prevented CCL2-induced inhibition of MOR-mediated analgesia.

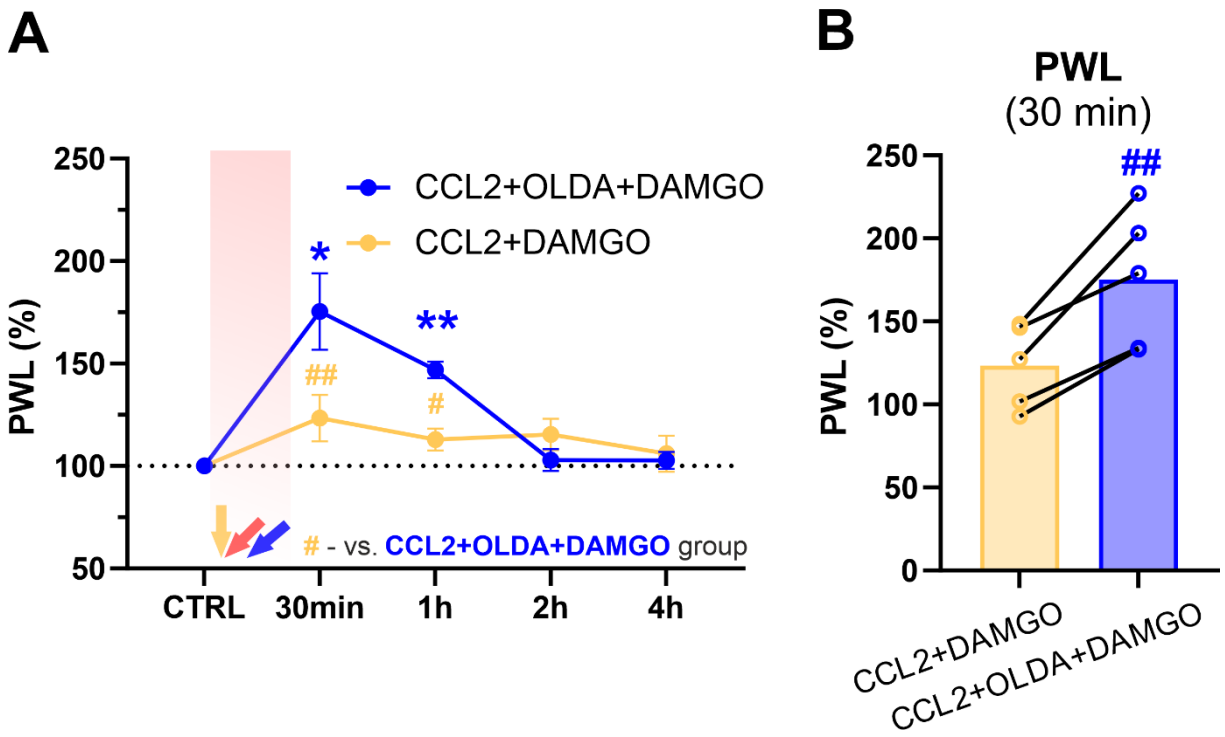

Figure 10.5.: **Intrathecal application of the endogenous TRPV1 agonist OLDA prevented CCL2-induced inhibition of MOR-mediated analgesia in the thermal plantar test.** (**A**) A single i.t. CCL2 (1.4 µg, n = 5) pre-treatment 30 min before the i.t. application of DAMGO was able to suppress MOR mediated analgesia; PWL remained statistically unchanged throughout the testing period (30 min: 123.3 ± 11.3%, P = 0.265; 1 h: 112.9 ± 5.4%, P = 0.265; 2 h: 115.4 ± 7.6%, P = 0.265; 4 h: 106.1 ± 8.8%, P = 0.265). Co-application of CCL2 with OLDA (0.42 µg) significantly attenuated CCL2-induced inhibition of MOR-mediated analgesia. (**B**) Comparison of PWL at peak effect time (30 min) after DAMGO. We assume that activation of TRPV1 by endogenous agonist OLDA prevented CCL2-mediated heterologous desensitization of MOR, leading to a decreased analgesic efficacy of DAMGO. Statistical differences in each experimental group were identified using RM ANOVA on ranks followed by Dunnett`s test; $^{*}P < 0.05$, $^{**}P < 0.01$, versus CTRL. Differences between treatments were analyzed using one-way ANOVA followed by the Bonferroni test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus CCL2 + OLDA + DAMGO group.

10.3. The role of PI3K and macrophages in the Paclitaxel-induced peripheral neuropathy

10.3.1 Paclitaxel in vivo treatment increased expression of pAkt in DRG neurons

As was discussed in Chapter 5, PI3K was shown to play an important role in the central sensitization in PIPN. To study PI3K activity, we looked at the level of Akt kinase phosphorylation, which reflects the PI3K signaling pathway activation (Zhuang et al., 2004; Pezet et al., 2008). Akt kinase phosphorylation at Ser473 phosphorylation site in L5 DRG neurons was determined using immunohistochemistry after *in vivo* PAC treatment (8 mg/kg; i.p.). Figure 10.6. shows representative images of pAkt immunofluorescent staining in L5 DRG sections under the control conditions (Fig. 10.6. A, B), after the PAC treatment (Fig. 10.6.D, E), and after the wortmannin pre-treatment (0.6 mg/kg, i.p.) followed by PAC (Fig. 10.6.G, H). Based on the histological data summarized in Figure 10.6., PAC significantly increased the pAkt positive area (Fig. 10.6.K) and the percentage of pAkt positive cells (Fig. 10.6.L). PAC-induced increase of both pAkt positive area and the number of pAkt positive neurons was prevented by a wortmannin pre-treatment (Fig. 10.6.C, F, I). In summary, PAC treatment induced increased phosphorylation of Akt, especially in the small-diameter neurons (< 25 μm) as the ratio between the pAkt positive and negative neurons increased after the PAC treatment most prominently in neurons of these size categories (Fig. 10.6.F), compared to the control and wortmannin + PAC groups (Fig. 10.6.C, I).

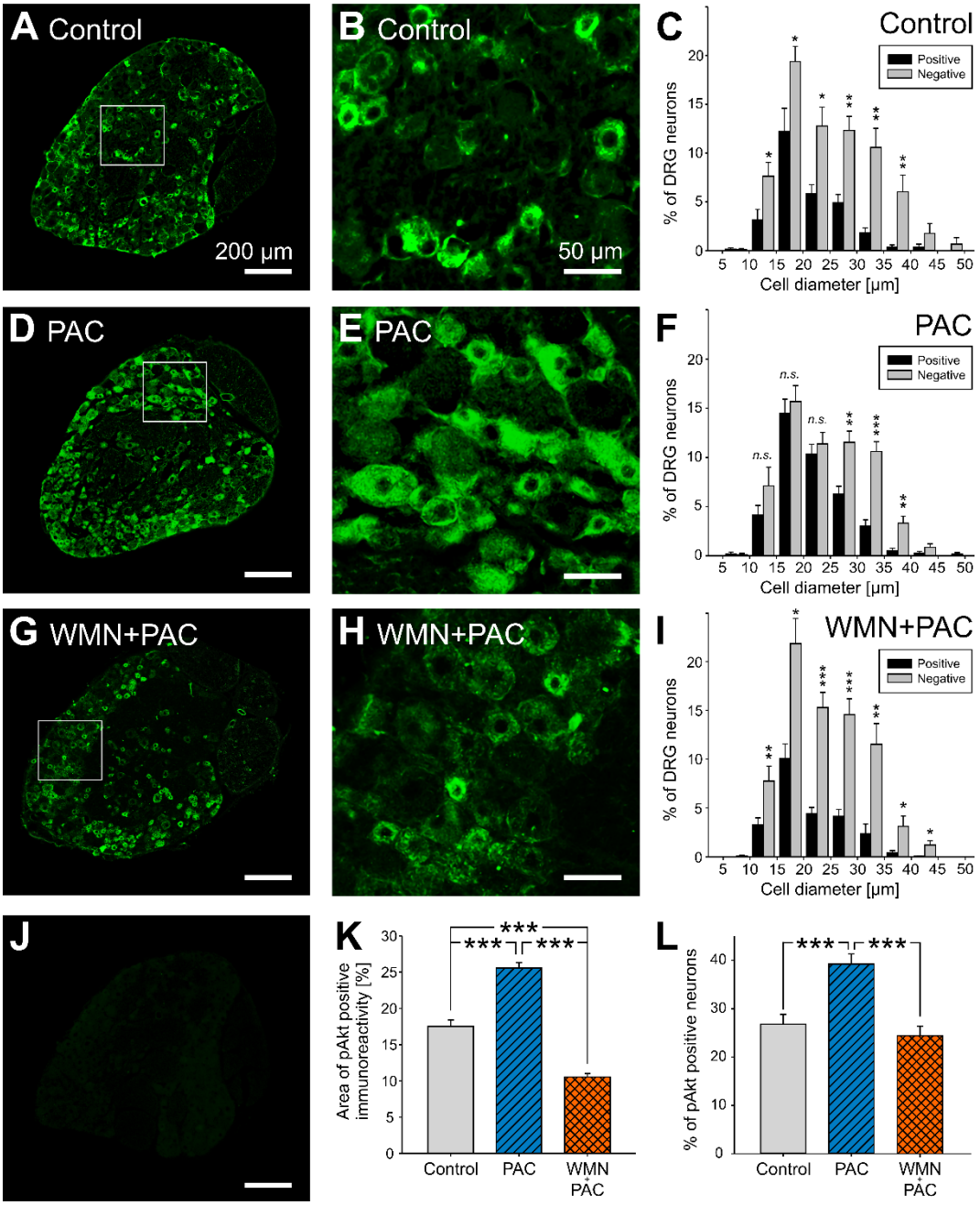

Figure 10.6.: **Wortmannin inhibited acute PAC administration-induced PI3K activity, evident as a decrease of Akt phosphorylation in L5 DRG neurons.** (**A, B**) A representative image of pAkt immunofluorescence in the DRG of the control group. (**C**) Size distribution of pAkt IR neurons shows prevalent pAkt presence in a population of small diameter neurons. (**D, E**) Acute PAC administration increased a pAkt IR area (**F**) as well as the relative number of pAkt IR small diameter neurons (< 25 µm). (**G, H**) Wortmannin pre-treatment (WMN + PAC) significantly reduced the PAC-induced pAkt IR area (**I**) as well as the relative number of $pAkt^{+}$ neurons, reverting Akt

phosphorylation to the control situation. (**J**) Representative image of DRG section incubated without primary antibody. (**K**) Comparison of IR^+ area in DRG slices in all three experimental groups; wortmannin pre-treatment prevented PAC-induced increase in pAkt IR. (**L**) PAC increased the relative number of $pAkt^+$ neurons and this was prevented by wortmannin. All data are represented as a mean with SEM. The paired t-test for each size group (**C, F, I**) or the One-Way ANOVA (**K, L**) was used. (*P < 0.05; **P < 0.01; ***P < 0.001).

10.3.2. DUV pretreatment prevented PAC-induced Akt phosphorylation

The following results report an effect of DUV, a novel FDA-approved PI3Kδ/γ isoform-specific inhibitor, in preventing PAC-induced pro-nociceptive changes in rat DRGs. We have studied this effect in DRG because both DUV and PAC have a limited blood-brain barrier (BBB) permeability (Yan et al., 2015; FDA, 2018), and BBB protection of DRGs is reported to be less effective in comparison to the CNS (Allen et al., 1994). As in previously described wortmannin + PAC experiments, the following results report the effect of DUV in preventing PAC-induced Akt phosphorylation in rat L5 DRGs using pAkt immunohistochemistry. Levels of pAkt were determined 1 h after the PAC (8 mg/kg, i.p.) administration. Vehicle administration was used in the CTRL group and the group labeled as 'DUV + PAC' received DUV (10 mg/kg, p.o.) pretreatment 30 min before the PAC. Acute PAC administration lead to a significant increase in pAkt IR area (Fig. 10.7.Ba, Bb), compared with the vehicle-treated CTRL (Fig. 10.7.Aa, Ab), while DUV pre-treatment prevented pAkt IR area increase (Fig. 10.7.Ca, Cb). The size distribution diagram of $pAkt^+$ neurons in the PAC group (Fig. 10.7.Bc) illustrates a significant increase of $pAkt^+$ neurons predominantly in the population of small diameter (15-25 μm) neurons compared with CTRL (Fig. 10.7.Ac) and DUV + PAC group (Fig. 10.7.Cc).

Previous results report changes after acute PAC treatment but mechanical allodynia induced in the experimental model of PIPN is present for up to 21 days. For this reason, we analyzed changes in pAkt IR in L5 DRG slices in timepoints relevant to observed behavioral hypersensitivity on days 7 and 21. Animals in the PAC group received three successive doses of 8 mg/kg PAC, i.p., on days 0, 3, and 6; DUV + PAC group received 10 mg/kg of DUV, p.o., 30 min pretreatment before the PAC and CTRL group received vehicle. The number of pAkt IR neurons was increased in the 7D PAC group (Fig.

10.7.E); a major increase was observed in the population of small-diameter neurons (20-25 μm) (Fig. 10.7.D) and DUV in 7D DUV + PAC group prevented PAC-induced IR increase (Fig. 10.7.F). No significant increase of pAkt IR was observed in the size distribution diagrams of DRGs collected on day 21 after the initial treatment (Fig. 10.7.G-I).

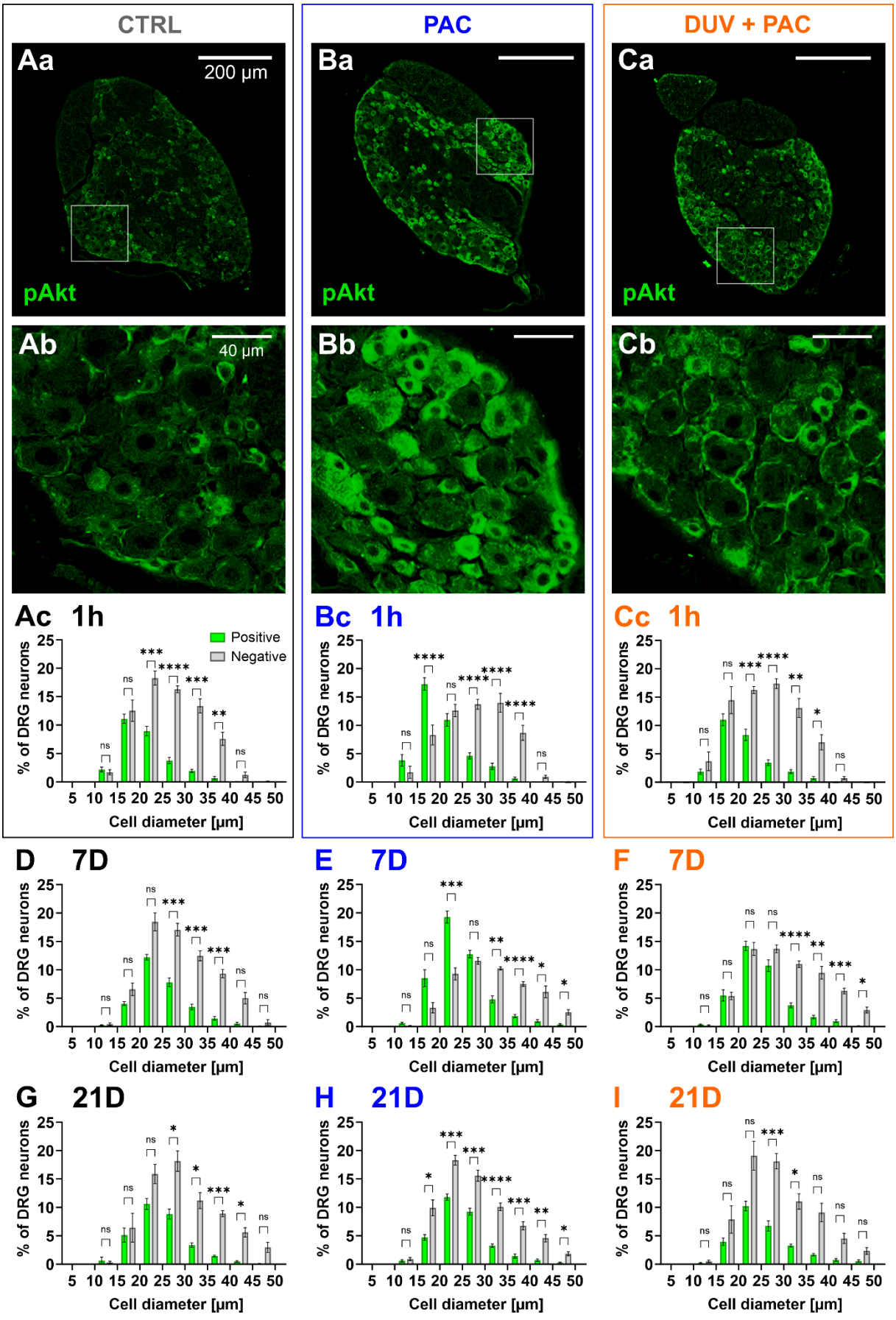
CTRL
PAC
DUV + PAC
Aa
200 μm
pAkt
Ba
pAkt
Ca
pAkt
Ab
40 μm
Bb
Cb
Ac 1h
Positive
Negative
% of DRG neurons
Cell diameter [μm]
Bc 1h
Cc 1h
D 7D
E 7D
F 7D
G 21D
H 21D
I 21D

Figure 10.7.: **PAC-induced increase of Akt phosphorylation in DRG neurons was prevented by DUV (10 mg/kg) pre-treatment *in vivo*.** (**Aa-Cc**) Representative images and a summary graph of the immunostained DRGs collected 1 h after the PAC (8 mg/kg, i.p.) treatment. (**Aa-Ac**) Representative images of pAkt IR in L5 DRG sections in the CTRL group and size distribution diagram (**Ac**) of the $pAkt^+$ and $pAkt^-$ neurons; pAkt is predominantly present in the population of small- and medium-diameter neurons. (**Ba-Bc**) The proportion of $pAkt^+$ small-diameter neurons in the category of 15-20 μm cells was significantly increased by an acute PAC treatment ($P < 0.001$); PAC significantly shifted the positive/negative ratio in the category of 20-25 μm cells ($P < 0.001$ in CTRL, vs $P = 0.9$ in PAC). (**Ca-Cc**) After the DUV p.o. pretreatment 30 min before the PAC i.p., the number of $pAkt^+$ cells was significantly reduced. (**D-I**) Size distribution of pAkt IR in 7D and 21D groups. (**D**) 7D CTRL: highest levels of pAkt IR cells were observed in the population of small- and medium-diameter neurons. (**E**) PAC significantly increased pAkt IR in small-diameter neurons (category of 20-25 μm cells) ($P < 0.001$) on day 7 of PAC treatment. (**F**) DUV pretreatment reduced the number of $pAkt^+$ small-diameter neurons on day 7. (**G-I**) The ratio of the $pAkt^+$ and $pAkt^-$ neurons in discrete size categories in CTRL, PAC, and DUV + PAC groups on day 21 revealed no significant differences. Two-way repeated-measures ANOVA followed by Bonferroni's post hoc multiple comparison test was used for comparing the differences in the percentage of positive cells in each diameter category ($P < 0.05$ to 0.001). Scale bars: **Aa**, **Ba**, **Ca**, 200 μm; **Ab**, **Bb**, **Cb**, 40 μm.

The size distribution of $pAkt^+$ neurons (Fig. 10.8.A) demonstrates significant increase of $pAkt^+$ neurons 1 h after PAC treatment (71.2 ± 0.8%; $P < 0.001$), in comparison with the CTRL group (28.8 ± 0.8%). This overall PAC-induced increase of $pAkt^+$ neurons was significantly reduced in the DUV + PAC group, reverting the number of $pAkt^+$ cells to the control situation (27.3 ± 0.8%). The same effect of PAC and DUV + PAC was reflected in the IR area analysis of the same L5 DRG slices (Fig. 10.8.B). The pAkt IR area was significantly increased by the acute PAC (28.6 ± 2.0%; $P < 0.05$) compared with the CTRL (21.3 ± 1.4%), while DUV pre-treatment was able to prevent PAC-induced increase (20.9 ± 1.6%). In DRGs collected on day 7 (Fig. 10.8.C), percentage of $pAkt^+$ neurons in PAC group was significantly higher than in 7D CTRL group (7D CTRL: 29.8 ± 1.5% ; 7D PAC: 49.1 ± 1.9% $P < 0.001$). DUV pre-treatment in the 7D DUV + PAC group reduced the number of $pAkt^+$ cells to 37.0 ± 1.6%. pAkt IR area in DRGs was

robustly increased in the 7D PAC group (Fig. 10.8.D) (37.6 ± 1.1%), compared with the 7D CTRL group (24.7 ± 1%; P < 0.001). DUV significantly reduced the increase of pAkt IR in the 7D DUV + PAC group (27.5 ± 1.3%). Only a single difference was observed on day 21 (two weeks after the last PAC treatment); the number of $pAkt^{+}$ cells in the 21D DUV + PAC decreased (27.3 ± 1.9%) in comparison with the 21D PAC (Fig. 10.8.E). No significant changes in the pAkt IR area were observed on day 21. In conclusion, our results show that DUV prevented PAC-induced increased activity of PI3K signaling in sensory DRG neurons, which can serve as one of the causes of sensory neuron sensitization at the spinal cord level. We have not observed any significant effect of PAC on pAkt IR on day 21 (2 weeks after the last treatment).

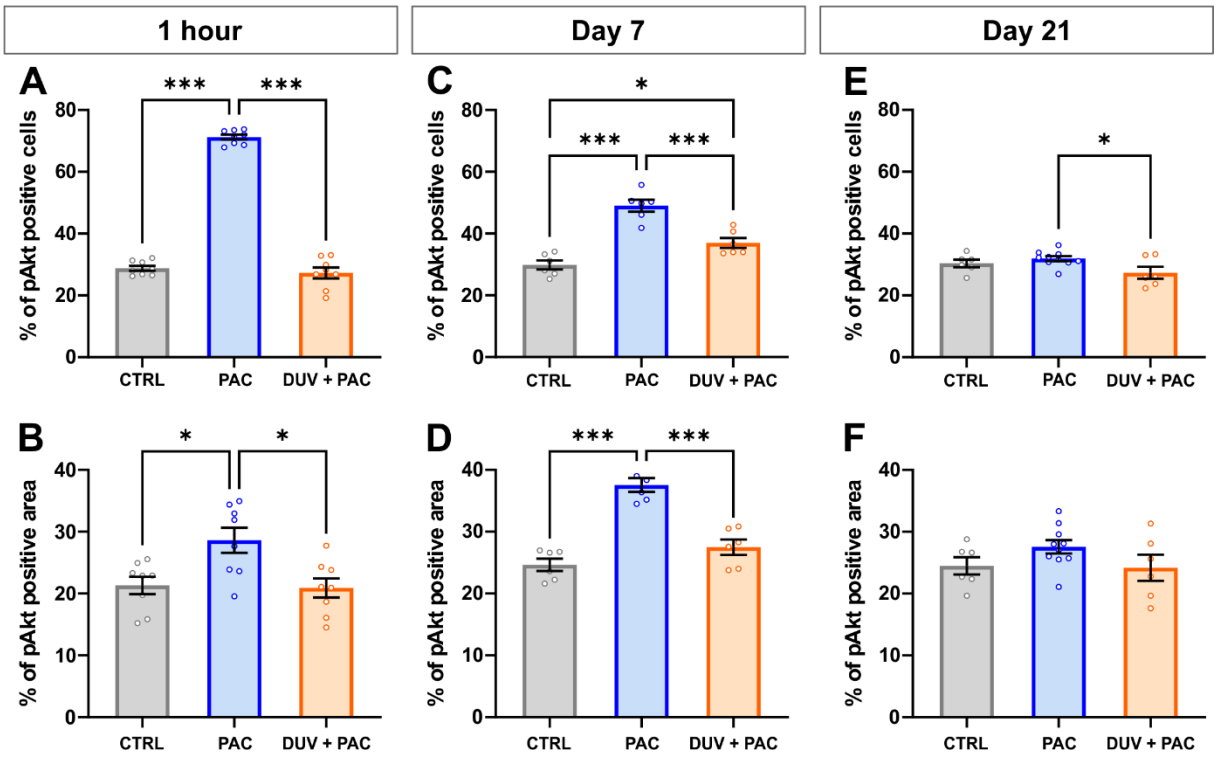

Figure 10.8.: **DUV pretreatment prevented PAC-induced Akt phosphorylation in DRG neurons.** (**A**) Acute administration of PAC significantly increased the percentage of the $pAkt^{+}$ neurons 1 h after the PAC administration; DUV pre-treatment prevented acute PAC-induced increase of Akt phosphorylation. Ordinary one-way ANOVA ($F(2,21)= 421.5$; $P < 0.001$) followed by Tukey's multiple comparison post hoc test ($P < 0.001$). (**B**) Acute administration of PAC significantly increased pAkt IR area; this effect was prevented by DUV pre-treatment. Ordinary oneway ANOVA ($F(2,21)= 6.601$; $P < 0.01$) followed by Tukey's multiple comparison post hoc test ($P < 0.05$). (**C**) 7D PAC (3 x

8 mg/kg, i.p.) induced an increase in the percentage of $pAkt^+$ cells; this increase was reduced by DUV pre-treatment. Ordinary one-way ANOVA ($F(2,15)= 33.61$; $P < 0.001$) followed by Tukey's multiple comparison post hoc test ($P < 0.05$ to 0.001). (**D**) $pAkt^+$ area was increased after PAC treatment on day 7; this increase was prevented by pretreatment with DUV. Ordinary one-way ANOVA ($F(2,15)= 36.22$; $P < 0.001$) followed by Tukey's multiple comparison post hoc test ($P < 0.001$). (**E, F**) No increase in the number of $pAkt^+$ neurons and pAkt IR area after PAC treatment was observed in DRG slices from animals on day 21. The only observed difference was a small decrease in the number of $pAkt^+$ cells in the 21D DUV + PAC group compared with 21 D PAC ($P < 0.05$). Ordinary one-way ANOVA ($F(2,19)= 3.56$; $P = 0.048$, for percentage of $pAkt^+$ cells; $F(2,19)= 1.83$; $P = 0.19$, for percentage of $pAkt^+$ area) followed by Tukey's multiple comparison post hoc test. For numbers of animals/DRGs/cells included in the analysis, see Methods.

10.3.3. DUV pretreatment prevented PAC-induced infiltration/ proliferation of CD68-immunoreactive macrophages in the rat DRGs

Infiltration of circulating monocytes and their differentiation to macrophages in the DRGs as well as proliferation from resident macrophages was reported to play an important role in the development and maintenance of neuropathic pain (Ji et al., 2016; Yu et al., 2020), including PIPN (Huang et al., 2014 b; Zhang et al., 2016). PI3K signaling in macrophages is important for the regulation of macrophage responses to metabolic and inflammatory signals and can shift macrophage polarization (Sharif et al., 2019). Additionally, the PI3Kδ isoform was demonstrated to regulate the infiltration and motility of macrophages (Mouchemore et al., 2013). For this reason, the experiment described in this chapter aimed to study if δ/γ isoform-specific inhibitor DUV can prevent the increase in macrophage infiltration/proliferation in the DRGs after PAC treatment. We have used immunofluorescent staining of CD68 lysosomal membrane protein, expressed predominantly in membranes of phagocytic macrophages (Damoiseaux et al., 1994), to analyze the distribution of macrophages in L5 DRG slices. We quantified CD68 IR area in adult rat L5 DRG sections from CTRL (vehicle-treated), PAC (3 x 8 mg/kg, i.p.), and DUV + PAC-treated animals (30 min pretreatment with DUV, 3 x 10 mg/kg, p.o. before PAC). L5 DRGs in all groups were collected 24 h after the last treatment (day 7 of the PIPN protocol). PAC significantly increased the CD68 IR (Fig. 10.9.B) in comparison

with the CTRL group (Fig. 10.9.A), while the DUV pretreatment (Fig. 10.9.C) prevented PAC-induced increase of the CD68 IR. Results are summarized in the Fig. 10.9 (Fig. 10.9.D), demonstrating an increase of $CD68^+$ area after the PAC treatments (2.0 ± 0.1% of DRG), a 43.6% increase compared with the CTRL group (1.4 ± 0.1% of DRG; $P < 0.001$); $CD68^+$ cells form an aggregates around neuronal cell bodies. DUV pretreatment significantly prevented the PAC-induced increase of CD68 IR (1.5 ± 0.1% of DRG; $P < 0.01$).

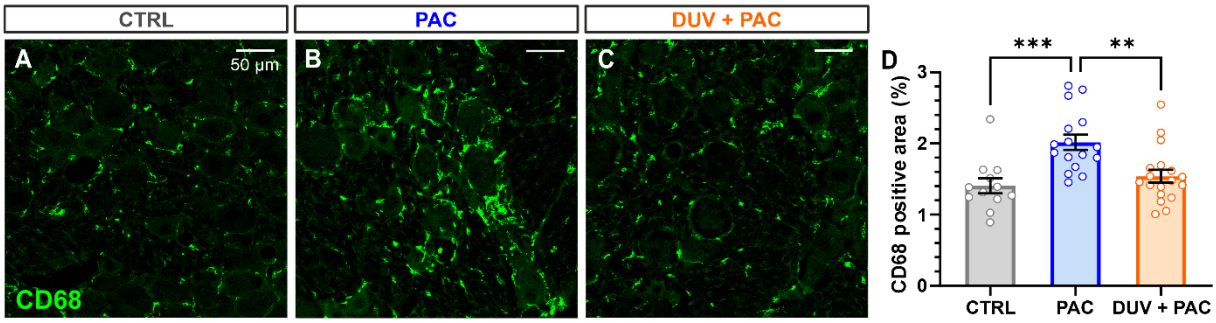

Figure 10.9.: **PAC-induced infiltration/proliferation of $CD68^+$ macrophages in the L5 DRGs was prevented by a DUV pre-treatment.** L5 DRGs used for immunohistochemistry were collected 24 h after the last treatment (day 7). PAC (3 x 8 mg/kg, i.p.) was administered on days 0, 3, and 6. DUV (3 x 10 mg/kg, p.o.) was administered as 30 min pretreatment before PAC. Representative images of CD68 immunohistochemical staining of L5 DRG sections from the (**A**) CTRL, (**B**) PAC, and (**C**) DUV + PAC treated animals. (**D**) PAC treatment significantly increased the CD68 IR area compared with the CTRL; PAC-induced infiltration/proliferation of macrophages was prevented by a DUV pre-treatment, as is evident by a decrease of CD68 IR in the DUV + PAC group. Ordinary one-way ANOVA ($F(2,43)= 9.729$, $P < 0.001$) followed by Tukey's post hoc test ($P < 0.01$ to 0.001). Numbers of animals/DRGs included in the analysis: CTRL (n = 6/12), PAC (n = 8/16), and DUV + PAC (n = 9/18). Scale bars: (**A-C**) 50 µm.

11. Discussion

11.1. DAMGO-induced depression of synaptic transmission and analgesia

Opioids hold a place as the most effective analgesics developed to date and are used to treat a wide variety of pain conditions from severe acute to chronic pain. However, clinical use of opioids is frequently limited by serious and often lethal side effects such as OIH, respiratory depression, constipation, risk of overdose, and addiction. Decades of research focused on mechanisms behind opioid-induced analgesia uncovered in great detail the molecular basis of opioid signaling both in peripheral tissues and in central nociceptive synaptic transmission, making progress toward more effective and safe opioid analgesics treatments.

Our experiments demonstrate DAMGO-induced inhibition of the spinal excitatory synaptic transmission and *in vivo* analgesia. Patch-clamp recordings from superficial dorsal horn neurons of lamina I and II_o reveal a significant DAMGO-induced depression of mEPSC's frequency and electrically evoked eEPSC amplitude. These results are in agreement with previous studies demonstrating excitatory synaptic depression in the spinal cord (Zhou et al., 2010; Heinke et al., 2011). The inhibitory effect of opioids in our experiments as well as in previous studies was mainly presynaptic, mediated by the terminals of Aδ- and C-type fibers. Opioids in these excitatory presynaptic terminals inhibit N-type and P/Q-type voltage-dependent Ca^{2+} channels and neurotransmitter release machinery (Zhou et al., 2010).

Relatively high levels of MORs in the spinal cord are distributed throughout the dorsal horn, expressed in both presynaptic terminals and postsynaptic spinal neurons (Besse et al., 1990; Ding et al., 1996). On the postsynaptic neurons, opioids activate GIRKs, which have the ability to decrease the excitability of spinal neurons (Nagi et al., 2014). However, opioids in analgesic concentrations failed to induce significant changes in the holding potential of the substantia gelatinosa neurons and to decrease the amplitude of inward excitatory currents (Wu et al., 2003). Finally, morphine retained its analgesic potency in GIRK2-null mice, with a maximal effect comparable to wild-type animals, highlighting that opioid-induced analgesia relies mostly on depression in presynaptic terminals (Mitrovic et al., 2003). This is in agreement with our results obtained from the

spinal cord, where DAMGO decreased the frequency of mEPSC without a change in amplitude, suggesting a dominant contribution of the presynaptic terminals modulation.

While all of the recorded neurons responded to the application of DAMGO by a rapid decrease of eEPSC amplitude, 60% of the neurons reached amplitude significantly higher than their respective control amplitudes during the washout period. This delayed potentiation of the excitatory synaptic transmission was first observed by Zhou et al., who also describes a similar effect on the frequency of mEPSC recordings in 50% of recorded neurons (Zhou et al., 2010). Although we have observed the delayed potentiation of mEPSC frequency only in 1 out of 16 recorded neurons, this discrepancy can be explained by a difference in the age of the animals (21 days old Wistar rats in our experiments; adult rats in Zhou et al.), recording temperature and a difference in subpopulations of the recorded neurons.

Recent findings shifted the research focus on the importance of pathways and receptors interfering with opioid signaling in an effort to identify pharmacological targets for improved opioid analgesia in a clinical setting. TRPV1 receptors emerged as a target, with the ability to prime opioid-induced analgesia and minimize the MOR desensitization through activation of the MAPK signaling pathway (Basso et al., 2019). Moreover, opioid-induced LTP in the spinal cord, in part responsible for OIH and opioid tolerance, has been shown to be restricted to a TRPV1 expressing primary afferents, as ablation of $TRPV1^{+}$ primary afferents eliminated the DAMGO-induced LTP (Zhou et al., 2010). As the TRPV1 in our experiments could be activated by endogenous agonists and changes in the membrane potential (Spicarova et al., 2009) and since the activation of MOR could lead to the TRPV1 sensitization through dissociation of the β-arrestin2 from MOR (Rowan et al., 2014), we have identified TRPV1 activation as a potentially crucial mechanism for the delayed DAMGO-induced synaptic potentiation.

To determine the functional effect of TRPV1-MOR interaction in our experiments, we repeated electrophysiological recordings from superficial dorsal horn neurons in the presence of a TRPV1 antagonist. While having no effect on DAMGO-induced inhibition of eEPSC amplitude, the TRPV1 antagonist completely prevented DAMGO-induced delayed potentiation. This result indicates that the potentiation we observed is dependent on the activation of TRPV1 in the primary afferent fibers. In our eEPSC recordings, electrical stimulation of the dorsal root stump evoked action potential propagation and

this change in presynaptic membrane voltage could contribute to the TRPV1 activation. Action potential propagation is absent during mEPSC recordings; the absence of membrane depolarization could explain why we have not observed the delayed potentiation in mEPSC recordings.

Results obtained from the behavioral experiments support the inhibitory actions of DAMGO on the excitatory synapse. Single i.t. administration of DAMGO lead to rapid and potent analgesia in the heat-induced paw withdrawal test. DAMGO administration in our experiments did not result in any signs of OIH that could reflect the delayed potentiation observed in eEPSC recordings. However, OIH is usually described after administration of opioids in doses higher than the dose of DAMGO used in our experiment or can be induced by an abrupt cessation of prolonged administration of fast-acting opioids (Drdla et al., 2009).

11.2. Chemokine CCL2 in opioid-induced analgesia

The efficacy of opioids in chronic pain treatment is severely hindered by the development of opioid tolerance, leading to a progressive increase in effective dose. While molecular and physiological mechanisms of opioids in CNS and PNS have been extensively studied for decades, our understanding of how changes in the spinal cord dorsal horn in chronic pain impact opioid analgesia is insufficient to combat these downsides.

Our results demonstrate a significant functional modulation of MOR signaling by a chemokine CCL2. We showed that the selective MOR agonist DAMGO induced fast and potent analgesia *in vivo* and significant inhibition of the excitatory synapse reflected as a decreased frequency of mEPSCs and amplitude of eEPSCs recorded from the SCDH neurons. Chemokine CCL2 prevented both DAMGO-induced analgesia and inhibition of excitatory synapse in a microglia-dependent manner, presumably through a MOR desensitization or internalization (Zhang et al., 2004).

An important role of CCL2 in neuropathic pain has been repeatedly demonstrated as a critical chemokine in nociceptor sensitization, leading to hypersensitivity in conditions such as neuropathic pain or neuroinflammation (Van Steenwinckel et al., 2011; Dansereau et al., 2021). An increase and presence of CCL2 in the sciatic nerve and DRG has been established in the CCI neuropathic pain model, together with the increase of CCR2 in DRG (Fu et al., 2010). A study on SNL neuropathic pain model described a

neuronal transport of CCL2 from DRG to the spinal cord and revealed an increase in CCL2 in the spinal cord that peaked with the development of the maximal mechanical allodynia (Jeon et al., 2009). Functional evidence of the ability of CCL2 to sensitize primary nociceptors and evoke hyperalgesia on its own comes from our previous study demonstrating how CCL2 can potentiate spontaneous glutamate release from the central endings of the nociceptive afferent fibers and induce thermal hyperalgesia and mechanical allodynia after single spinal application (Spicarova et al., 2014). The results presented here expand these findings by providing evidence for the role of CCL2 in the dysregulation of opioid-induced analgesia. Sensitization of nociceptive pathways and inhibition of opioid signaling at the same time might have a synergistic effect on the development of hypersensitivity. This finding has important implications for (1) understanding of neuropathic chronic pain onset and (2) low efficacy of opioids in neuropathic pain treatment.

Our findings of CCL2-induced attenuation of opioid-induced analgesia and inhibition of nociceptive transmission in the spinal cord are in alignment with the previous studies, showing chemokine-induced MOR internalization in cell cultures (Zhang et al., 2004). Cell culture experiments show how CCL2 as well as CCL3, CCL5, and CXCL8 impair DAMGO-elicited calcium response and induce internalization of MOR in MOR-expressing cells, indicating heterologous desensitization of MOR.

11.3. Microglia are crucial for CCL2-induced desensitization of MOR

Results presented in this thesis indicate an essential role of microglia in CCL2-induced inhibition of opioid analgesia. Microglia in SCDH maintain frequent communication with neurons and are known to be an important element in the modulation of neuronal excitability and nociceptive signaling. Blocking the activation of microglia by minocycline prevented the inhibitory effect of CCL2 on MOR in eEPSC recordings and *in vivo* analgesia, without a significant effect on DAMGO potency. Despite the uncertainty regarding the distribution of CCL2 and CCR2 in spinal cord cell types stemming from contradictory results in older literature, our results suggest that activation of microglia is a crucial event in the CCL2-induced desensitization of MOR.

Past studies report the expression of CCR2 mRNA in cultured neurons (Gosselin et al., 2005) and the direct effect of CCL2 on spinal neurons in acute spinal cord slices (Spicarova et al., 2014). A different study utilized immunohistochemistry and reported the presence of CCR2 protein exclusively in spinal astrocytes (Kawaguchi-Niida et al., 2014). Surprisingly, data obtained from transgenic CCR2-reporter line show no detectable CCR2 in the lumbar spinal cord of healthy mice, and a substantial expression of CCR2 in infiltrating monocytes and activated microglia during the progression of amyotrophic lateral sclerosis (Komiya et al., 2020). Moreover, blocking the activation of microglia by minocycline was shown to inhibit CCL2-induced hyperalgesia and potentiation of the spinal excitatory transmission, connecting the effects of CCL2 on the spinal cord neurons with the activation of microglia (Huang et al., 2014 a).

Controversies and parallels mentioned above led us to consider the role of microglia in the effects we reported both *in vitro* and *in vivo*. Although we did not reveal detailed mechanisms of observed effects, it is possible that microglia amplify the CCL2-induced desensitization of MOR by the release of pronociceptive mediators such as IL-1β, IL-6, and IL-18 (Kwiatkowski et al., 2017). In addition, minocycline failed to prevent the CCL2-induced effect on DAMGO-induced depression of mEPSC frequency. Based on this finding we assume that CCL2 can attenuate DAMGO-induced depression of spontaneous glutamate release, recorded in our case as the frequency of mEPSC, directly by acting on presynaptic neurons. This, however, does not invalidate the importance of microglia in the CCL2-induced desensitization of MOR but rather highlights the complexity of the MOR signaling modulation. The role of microglia is evident in eEPSC recordings and behavioral experiments, which reflect action potential mediated glutamate release, where direct action of CCL2 on presynaptic neurons was insufficient to attenuate CCL2-induced effects on MOR and microglia played a crucial part.

11.5. OLDA prevents both agonist-induced and chemokine-induced inhibition of MOR-mediated opioid effects

A rising number of studies recently highlighted the importance of MOR and TRPV1 interaction in inflammation and endogenous opioid-mediated analgesia (Basso et al., 2019), development of OIH (Heles et al., 2021; Wang et al., 2022), and regulation of MOR phosphorylation (Scherer et al., 2017). MORs are co-expressed with TRPV1 on central terminals of primary afferent neurons in the spinal cord dorsal horn. Activation of

TRPV1 at these presynaptic endings promotes MAPK signaling pathway, leading to the β-arrestin2 trafficking to the cell nucleus. This exclusion of β-arrestin2 from cytosol prevents its binding to the MOR and thus leads to attenuated MOR desensitization (Basso et al., 2019).

Our study focused on the interaction of TRPV1 and MOR using endogenous agonists of TRPV1 receptors OLDA and revealed that activation of TRPV1 can modulate both agonist-induced and chemokine-induced desensitization of MOR *in vivo* and in acute spinal cord slices *in vitro*. Patch-clamp recordings from acute spinal cord slices show significant DAMGO-induced desensitization of MOR, that was prevented by OLDA in a concentration-dependent manner. Therefore, activation of TRPV1 by its endogenous agonist OLDA is sufficient to minimize MOR desensitization. To see whether the attenuation of MOR desensitization can translate into enhanced analgetic efficacy of opioids, we used *in vivo* CCL2-induced MOR desensitization paradigm from our previous study (Heles et al., 2021). In this experiment, i.t. CCL2 induced MOR desensitization evident as a lack of thermal analgesia after i.t. DAMGO application. Based on previous *in vitro* studies exploring pathways activated by chemokine receptors that lead to MOR internalization, we assume that this attenuation of analgesia is caused by heterologous desensitization of MORs (Kwiatkowski et al., 2019). OLDA administered together with CCL2 prevented MOR desensitization as subsequent i.t. DAMGO administration evoked thermal analgesia comparable to the control situation, and thus confirmed that the activation of TRPV1 by its endogenous agonist can promote opioid analgesia in conditions that lead to elevated levels of chemokines in the spinal cord, such as chronic neuropathic pain. In summary, our data reveal how endogenous TRPV1-mediated pathways may interact with MOR function, reduce MOR desensitization, and in turn promote the efficacy of opioids in chronic pain. Further study of the underlying mechanisms of the TRPV1-β-arrestin2 signaling axis could contribute to improved opioid-mediated analgesia.

11.6. PI3K in Paclitaxel-induced neuropathic pain

PIPN is a frequent side effect of PAC chemotherapy, often accompanied by chronic neuropathic pain that negatively impacts the quality of a patient's life and can be a dose-limiting step of the therapy (Reyes-Gibby et al., 2009; Seretny et al., 2014). Recent research identified pro-nociceptive modulation in the DRGs by PAC, dependent

on sensitization of TRPV1$^+$ nociceptors (Li et al., 2015; Adamek et al., 2022). Moreover, the imbalance in nociceptive transmission evoked by a PAC treatment is further enhanced by disinhibition within the SCDH. PAC led to a reduction of the glycinergic component of the inhibitory neurotransmission in the SCDH, thus shifting the balance of the nociceptive transmission further towards excitatory signaling (Adamek et al., 2022). Results reported in this thesis are a part of the study which identified PI3K signaling as a crucial pathway in PIPN development and the accompanying chronic neuropathic pain (Adamek et al., 2022).

The most important finding of the study is that the oral administration of PI3Kδ/γ isoform-specific inhibitor DUV prevented chronic pain development and attenuated pro-nociceptive signaling and disinhibition in the PIPN model. The ability of the activated PI3K signaling pathway to sensitize TRPV1 receptors and TRPV1-mediated responses has been previously demonstrated as an increase in TRPV1 expression (Zhang et al., 2005) and an increase in capsaicin responses in DRG neurons (Zhu et al., 2007 b). Results from our studies demonstrate sensitization of TRPV1 in SCDH as a loss of capsaicin-mediated tachyphylaxis after both acute as well as 7 day PAC treatment (Adamek et al., 2019, 2022). Interaction between TRPV1 and PI3K is mediated by a catalytic subunit p85 of PI3K, which can bind to an ankyrin repeat domain of TRPV1 to increase its trafficking to the plasmatic membrane. Subsequent higher incorporation of TRPV1 receptor in the plasmatic membrane of the presynaptic endings of the nociceptors can lead to increased responsiveness to TRPV1 agonists like capsaicin and central sensitization (Stein et al., 2006; Stratiievska et al., 2018). Both TRPV1 sensitization recorded *in vitro* and mechanical allodynia *in vivo* were prevented by PI3K inhibitors LY-294002 and wortmannin (Adamek et al., 2019) and by PI3Kδ/γ isoform-specific inhibitor DUV (Adamek et al., 2022).

Immunohistological analysis of pAkt distribution in slices obtained from lumbar DRGs supports our findings and demonstrates that PI3K inhibitors wortmannin and DUV prevented PAC-mediated increase in pAkt distribution. We have selected phosphorylation of Akt kinase as a marker of increased PI3K signaling pathway activity. An increase in Akt kinase phosphorylation mediated by a PI3K signaling pathway was previously demonstrated in other inflammatory and neuropathic pain models (Xu et al., 2007, 2014; Pezet et al., 2008).

An increase in pAkt positivity, measured as a pAkt IR area in L5 DRG slices, was observed 1 h after a single administration of PAC (8 mg/kg, i.p.), showing that PI3K signaling is activated shortly after PAC administration and supporting role of PI3K signaling in observed acute effects of PAC. Similar to area increase, a relative number of $pAkt^+$ neurons in DRG increased after PAC treatment. Moreover, analysis of the pAkt distribution in neurons of various diameters revealed that the increase of pAkt was most prominent in small diameter neurons, in categories of neuron soma diameters between 15 to 25 μm. The increase of pAkt in small diameter neurons is in alignment with the PAC-mediated activation of TLR4 and potentiation of TRPV1, as both TLR4 and TRPV1 are in DRGs expressed mostly in small diameter neurons.

Single acute pretreatment by either wortmannin or DUV prevented PAC-induced increase in pAkt phosphorylation 1 hour after PAC application. The level of Akt phosphorylation after WMN + PAC and DUV + PAC, measured as a ratio of $pAkt^+$ area in the DRG slice, decreased to a level comparable to the CTRL group. In addition to the pAkt IR area, a relative number of $pAkt^+$ neurons was decreased by both wortmannin and DUV. Based on the size distribution of $pAkt^+$ neurons in DRGs, Wortmannin and DUV prevented the increase of pAkt immunoreactivity in small and medium diameter neurons, reversing the size distribution diagram to a situation similar to CTRL.

To obtain results relevant to our behavioral experiments, we have measured the level of Akt phosphorylation in rat L5 DRG slices collected on day 7 and day 21 from animals undergoing treatment identical to a treatment regiment of animals used for *in vivo* behavioral experiments. First, PAC-treated animals show elevated levels of PI3K activity on day 7 of the treatment, evident by an increase of pAkt IR area compared to the CTRL animals. In this case, DRGs were collected 24 h after the last treatments. No significant change in the pAkt IR area was observed in the PAC group vs. CTRL on day 21, 14 days after the last treatment. Second, DUV prevented PI3K activity on day 7, evident by levels of pAkt IR area comparable to the CTRL group. These data imply that DUV can effectively suppress the effect of PAC on PI3K activity not only transiently, 1 h after the treatment, but throughout the development of PIPN at the timepoints where we observed the PAC-induced effects on TRPV1 sensitization and mechanical allodynia.

11.7. Effect of Duvelisib on PAC-induced infiltration/proliferation of macrophages in DRGs

Our experiments aimed at DUV's ability to prevent PAC-induced mechanical allodynia showed that DUV can also prevent the PAC-induced increase of macrophage infiltration/proliferation in rat DRGs. Infiltration of macrophages to DRGs after the PAC treatment is induced by a PAC-induced increase of chemoattractants, especially CCL2 and CX3CL1 (Huang et al., 2014 b; Zhang et al., 2016). Infiltration of circulating macrophages and activation of resident macrophages leads to increased production and release of proinflammatory cytokines and chemokines, such as TNFα and IL-1β, which in turn increase the excitability of DRG neurons (Spicarova et al., 2011; Ji et al., 2016) and promote sensitization of TRPV1 (Spicarova et al., 2014).

Active macrophages can be classified into M1 and M2 phenotypes and PAC, as well as DUV, promote a switch toward the M1 macrophage phenotype, possibly through PI3K signaling (Malyshev et al., 2015; Horwitz et al., 2018; Wanderley et al., 2018). Although the M1 macrophage phenotype is considered pro-inflammatory and pro-nociceptive, DUV was also shown to inhibit colony-stimulating factor 1 (CSF1), an important signaling factor for macrophage infiltration. CSF1 was shown to be increased neuropathic pain (Guan et al., 2016) and to attract circulating monocytes to the spinal cord and DRGs (Peng et al., 2016). Our claim on macrophage infiltration/proliferation inhibition is based on a decrease of CD68 immunoreactivity in DRGs and could contribute to the DUV-mediated antinociceptive effect by suppressing PAC-activated macrophages.

12. Conclusion

Modulation of synaptic transmission in superficial dorsal horn neurons has a crucial role in nociceptive signalization. During acute, chronic, and neuropathic pain conditions different mechanisms can induce changes in the SCDH and DRG that promote not only pro-nociceptive sensitization of the first nociceptive synapse but can also impair opioid-induced analgesia. Among such factors with particular importance in the development of neuropathic pain are chemokine CCL2 and TRPV1 receptors. Modulation of nociceptive signaling by these factors was therefore selected as the main focus of the first part of the results presented in this thesis. The second part of the presented results focused on changes in DRG leading to painful neuropathy in a model of chemotherapy-induced neuropathic pain. The goal was also to determine the possible use of a clinically approved inhibitor of PI3K inhibitor (Duvelisib) for PIPN treatment.

Our experiments clearly showed that MOR activation by the specific agonist DAMGO induced robust analgesia *in vivo* and inhibition of excitatory nociceptive transmission *in vitro*. This analgesic and inhibitory actions of DAMGO were potently diminished by treatment with chemokine CCL2 and this effect was highly dependent on microglia activation. It was shown before that the levels of CCL2 are increased during various, especially neuropathic pain conditions. Our results thus suggest that modulation of nociceptive synaptic transmission in the SCDH by CCL2 may be one of the reasons for decreased analgesic efficacy of opioids during these pathological states (Heles et al 2021). In this respect, equally important are our findings that in a model situation the signs of OIH were reduced by TRPV1 receptors antagonist. It was suggested before that TRPV1 may play a crucial role in MOR desensitization, through the regulation of β-arrestin 2 translocation (Basso et al., 2019; Melkes et al., 2020). Our unpublished data presented in this thesis show that activation of TRPV1 receptors with its endogenous agonist OLDA prevented the reduction of DAMGO effects after agonist-induced and CCL2-induced MOR desensitization.

Chemotherapy-induced chronic neuropathic pain states are still very difficult to treat and represent a severe burden for the patients, often leading to interruption of the chemotherapy treatment. In our experiments, we have demonstrated a significant role of PI3K activation in the process of PIPN development. We have shown in a PIPN model of CIPN a significant increase in PI3K activity as well as macrophage

infiltration/proliferation in the DRG (Adamek et al., 2019). Our results also clearly demonstrate that specific PI3K inhibitor DUV significantly attenuated both the PI3K activity and macrophage infiltration/proliferation. Results presented in the thesis are aligned with the rest of the experiments including behavioral testing and recordings of SCDH neurons activity, strongly suggesting that this clinically available PI3K antagonist DUV could be a good potential target for PIPN treatment (Adamek et al., 2022).

Overall, the results presented in this doctoral thesis show new potential mechanisms of neuropathic pain states development and highlight TRPV1 as an important target for the potential development of improved opioid analgesics and substantiate the key role of the chemokines in the limited efficacy of opioids in neuropathic pain. Hopefully, data published based on this work will provide a basis for future research and novel treatments of neuropathic pain that would help the patients.

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Appendix

List of publications related to the doctoral thesis:

Heles M, Mrozkova P, Sulcova D, Adamek P, Spicarova D, Palecek J. Chemokine CCL2 prevents opioid-induced inhibition of nociceptive synaptic transmission in spinal cord dorsal horn. *Journal of Neuroinflammation.* 2021 Dec 2;18(1):279. doi: 10.1186/s12974-021-02335-4.

(**IF = 9,587**, Quartile 1, Times Cited: 1, 8/2022 from WoS)

My personal contribution to this paper includes performing of majority of the electrophysiological recordings, implantation of intrathecal catheters, and performing of all behavioral experiments included in the study. I also contributed to the evaluation and statistical analysis of the data, and to the writing of the manuscript.

Adamek P, **Heles M**, Bhattacharyya A, Pontearso M, Slepicka J, Palecek J. Dual PI3Kδ/γ Inhibitor Duvelisib Prevents Development of Neuropathic Pain in Model of Paclitaxel-Induced Peripheral Neuropathy. *Journal of Neuroscience.* 2022 Mar 2;42(9):1864-1881. doi: 10.1523/JNEUROSCI.1324-21.2021.

(**IF = 6,709**, Quartile 1, Times Cited: 1, 8/2022 from WoS)

My personal contribution to this paper includes performing all of the immunohistochemistry, including tissue sample collection, image acquisition and analysis. I also participated in drug treatment protocols for behavioral studies and in the writing of the manuscript.

Adamek P, **Heles M**, Palecek J. Mechanical allodynia and enhanced responses to capsaicin are mediated by PI3K in a paclitaxel model of peripheral neuropathy. *Neuropharmacology*. 2019 Mar 1;146:163-174. doi: 10.1016/j.neuropharm.2018.11.027.

(**IF = 4,249**, Quartile 1, Times Cited: 15, 8/2022 from WoS)

My personal contribution to this paper includes performing all of the immunohistochemistry, including tissue sample collection, image acquisition and analysis. I also participated in drug treatment protocols for behavioral studies.