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Neuroinflammation and mechanisms of neuropathic pain development

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Abstract

Neuropathic pain represents a possible outcome of neural tissue injury; it occurs also as a concomitant symptom of different diseases or as a side effect of several treatments. Up to date, it constitutes a great challenge in clinical practice, as currently available treatments are still unsatisfactory. Mechanism-based treatment approaches are promising strategy in neuropathic pain management. However, there is still a lack of information about the exact mechanisms involved in the development and/or maintenance of neuropathic pain.

This Doctoral Thesis is aimed to explore the mechanisms underlying the development of neuropathic pain states in different models. The principal part of this work is focused on the study of anti-inflammatory effect of Angiotensin II receptor type 1 (AT1R) blocker, losartan, in two different models of peripheral neuropathy: paclitaxel-induced peripheral neuropathy (PIPN) and spinal nerve ligation (SNL). The work also aimed to access the involvement of spinal transient receptor potential vanilloid type 1 (TRPV1) channels in the process of neuronal activation induced by paclitaxel (PAC) and chemokine CCL2 treatment.

In order to fulfil the abovementioned aims, behavioral, immunohistochemical and molecular methods were used. For every model of peripheral neuropathy, the behavioral responses to thermal/mechanical stimuli were tested as a measure of increased pathological sensitivity - allodynia and hyperalgesia. Immunohistochemical methods were used to evaluate enhanced neuronal activation in the spinal cord dorsal horn (SCDH) and macrophage invasion in the dorsal root ganglia (DRGs). Western blot, ELISA, and RT PCR were used to determine the expression of specific proteins and mRNAs in SCDH and DRGs.

Our results demonstrate analgesic and anti-inflammatory effects of systemic treatment with losartan in the SNL and PIPN models of neuropathy. In both these models, losartan treatment, presumably through peroxisome proliferator-activated receptors gamma (PPARγ) agonism, attenuated the development of neuropathic pain and suppressed the expression of pro-inflammatory markers: CCL2, TNFα, CD11b, CD68, and others. Moreover, in the PIPN model, losartan treatment induced the expression of pro-resolving markers,

indicating the possible approach for the modulation of neuroinflammation. Our results also indicate active role of the spinal TRPV1 receptors in the mechanisms of central sensitization, as blockade of these receptors prevented increased activation of dorsal horn neurons in spinal cord slices, incubated with cytostatic PAC or chemokine CCL2. Moreover, TRPV1 antagonist intrathecal treatment prevented CCL2-induced thermal hyperalgesia in rats.

Studying mechanisms underlying the development of neuropathic pain is essential for the elaboration of new effective analgesic treatments. This work brings new information that may help to understand the complexity of neuropathic pain pathophysiology, and reveals new evidence about the mechanisms underlying the development of neuroinflammatory changes in the DRGs and spinal cord.

Abstrakt

Neuropatická bolest se nejčastěji objevuje po poškození nervové tkáně a také jako doprovodný příznak u řady onemocnění nebo jako nežádoucí vedlejší účinek některých cytostatických léčiv. V současné době neuropatická bolest představuje významný klinický problém, jelikož aktuálně dostupná analgestická léčba je stále neuspokojivá. Pro rozvoj nových účinných léčebných postupů neuropatické bolesti je důležité odhalit mechanizmy jejího vzniku. Stále však chybí přesné informace o procesech, které se podílejí na vývoji a udržování neuropatické bolesti.

Cílem této dizertacní práce bylo prozkoumat mechanizmy zapojené do vzniku neuropatických bolestivých stavů u několika modelů. Hlavní část práce je zaměřená na studium protizánětlivého účinku blokátoru angiotenzinových receptorů typu 1 (AT1R), losartanu, u dvou již etablovaných modelů periferní neuropatie a to navozené podáním cytostatika paclitaxelu (PIPN) nebo podvázáním míšního nervu (SNL). Další část této práce je věnována zkoumání úlohy vaniloidních receptorů typu 1 (TRPV1) u procesů aktivace míšních neuronů pod vlivem aplikace chemoterapeutika paclitaxelu nebo chemokinu CCL2.

Pro dosažení postavených cílů byly použity jak behaviorální, tak i molekulárně biologické metody. U každého modelu byly měřeny odpovědi potkanů na tepelné/mechanické podněty, které indikují míru zvýšené patologické citlivosti k těmto podnětům (alodynie a hyperalgezie). Imunohistochemické metody byly využity pro stanovení míry zvýšené aktivace neuronů v zadním rohu míšním (SCDH), a také pro vyhodnocení počtu cirkulujících makrofágů vstupujících do dorzálních ganglií (DRG). K vyhodnocení exprese specifických proteinů a mRNA v DRG a SCDH byly použity metody western blot, ELISA a RT PCR.

Naše výsledky ukázaly analgetické a protizánětlivé účinky systémového podání losartanu u SNL a PIPN modelů neuropatie. U obou zmíněných modelů léčba losartanem, pravděpodobně prostřednictvím agonizmu receptorů aktivovaných proliferatory peroxizomů typu γ (PPARγ), dokázala potlačit rozvoj neuropatické bolesti a snížit expresi prozánětlivých markerů CCL2, TNFα, CD11b,

CD68 a dalších. U PIPN modelu léčba losartanem navíc způsobila zvýšení exprese protizánětlivých markerů, což naznačuje možný způsob modulace neurogenního zánětu. Naše výsledky ukazují také aktivní úlohu míšních TRPV1 receptorů v mechanizmech centrální senzitizace. Zablokování těchto receptorů zabránilo zvýšené aktivaci neuronů v míšních řezech, inkubovaných s paclitaxelem nebo chemokinem CCL2. Intratekální aplikace TRPV1 antagonisty navíc zabránila vzniku akutní tepelné hyperalgézie vyvolané podáním CCL2.

Studium mechanizmů vzniku neuropatické bolesti je zásadní pro vývoj nových analgetických léčebních postupů. Tato práce přinesla nové poznatky, které mohou přispět k pochopení patofyziologických pochodů u neuropatických stavů a odhaluje nové informace o mechanizmech rozvoje neurogenního zánětu na úrovni míchy a DRG.

Contents

List of Abbreviations

ACE - angiotensin converting enzyme
ALS - anterolateral system
AMPA - α-amino-3 hydroxy-5-methyl-4-isoxazole propionate
Ang II – angiotensin II
ANOVA – analysis of variances
AP-1 - activator protein 1
ARB - angiotensin receptor blocker
ARG1 – Arginase 1
ATP - adenosine triphosphate
AT1R - Angiotensin II receptor type 1
AT2R - Angiotensin II receptor type 2
BBB - blood-brain barrier
BNB - blood-nerve barrier
BSCB - blood-spinal cord barrier
CaMKII - Ca2+/calmodulin-dependent protein kinase II
CAS – The Czech Academy of Sciences
CCI - chronic constriction injury
CCL - C-C chemokine ligand
CCL2 - C-C motif ligand 2
CCR - C-C chemokine receptor
CCR2 – C-C chemokine receptor type 2
CD11b – cluster determinant 11b, OX42, integrin αM
CD64 - Fc receptor that binds monomeric IgG-type antibodies
CD68 – cluster of differentiation 68, a phagocytic marker of monocyte lineage cells
CD163 - scavenger receptor for the hemoglobin-haptoglobin complex
CD206 - phagocytic mannose receptor C type 1
CGRP - calcitonin-gene related peptide
CIPN - chemotherapy-induced peripheral neuropathy
CNS - central nervous system
COX2 – cyclooxygenase type 2
CSF - cerebrospinal fluid
CSF1 - colony-stimulating factor 1
CTRL – control
CX3CR1 – CX3C chemokine receptor 1 or fractalkine receptor DRG - dorsal root ganglion

DAB - 3,3'-diaminobenzidine

DH – dorsal horn

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DR – dorsal root

ECF - extracellular fluid

ELISA - enzyme-linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

GABA - gamma-aminobutyric acid

GDNF - glial-derived neurotrophic factor

gDR – dorsal root segment that is closer to the DRG

GFAP - glial fibrillary acidic protein

HT – high threshold neurons

IASP - International Association for the Study of Pain

IFNγ - interferon gamma

IgG - immunoglobulin G

IL1β - interleukin 1 beta

IL1ra - interleukin-1 receptor antagonist

IL4 – interleukin 4

IL6 - interleukin 6

IL8 – interleukin 8

IL10 – interleukin 10

IL13 – interleukin 13

iNOS - inducible nitric oxide synthase

i. p. – intraperitoneal administration

IR – immunoreactivity

IRF4 - interferon regulatory factor 4

IRF5 - interferon regulatory factor 5

i.t. – intrathecal administration

JAK – Janus kinase

KCC2 - K+-Cl- cotransporter

LPS – lipopolysaccharide

LT – low threshold neurons

MAP - ERK mitogen-activated protein kinase

MCP-1 - monocytes chemoattractant protein 1, CCL2

mRNA – messenger ribonucleic acid

Nav1.9 - tetrodotoxin-resistant voltage-gated sodium channel type 1.9

NDS - normal donkey serum

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NGF - nerve growth factor

NMDA - N-methyl-D-aspartate

nNOS - neuronal nitric oxide synthase

NO - nitric oxide

NOS - nitric oxide synthase

NOX2 - nicotinamide adenine dinucleotide phosphate oxidase isoform

NSAID - nonsteroidal anti-inflammatory drug

OX42 – CD11b, integrin αM

PAC – paclitaxel

PAG - periaqueductal gray

PAF - primary afferent fibers

PB – phosphate buffer

PBS – phosphate-buffered saline

PEA – palmitoylethanolamide

pERK - phosphorylated extracellular signal-regulated kinase

PIPN – paclitaxel-induced peripheral neuropathy

PIP2 - phosphatidylinositol 4, 5-bisphosphate

PKA - protein kinase A

PKC – protein kinase C

PLC – phospholipase C

PNS - peripheral nervous system

p.o. – per os, oral administration, systemic administration

PPAR - peroxisome proliferator-activated receptor

PPARγ - peroxisome proliferator-activated receptor gamma

PPRE - peroxisome proliferator-activated receptor response element

PWL - paw withdrawal latency

PWT - paw withdrawal threshold

P2X4 - P2X purinergic receptor 4

RAS - renin-angiotensin system

RM ANOVA - repeated measures analysis of variance

RNA - ribonucleic acid

ROI - region of interest

ROX - reactive oxygen species

RT – room temperature

RT-PCR – reverse transcription - polymerase chain reaction

RVM - rostral ventromedial medulla

RXR - retinoid X receptor

SABC – streptavidine-biotine conjugation

SCDH - spinal cord dorsal horn

scDR – dorsal root segment that is closer to the spinal cord

SDS-PAGE - sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SEM – standard error measurement

SGC – satellite glial cell

SNL - spinal nerve injury

STAT1 - signal transducer and activator of transcription 1

STAT6 - signal transducer and activator of transcription 6

STT - spinotalamic tract

S100β – glial calcium-binding peptide

TBS - Tris-buffered saline

TGFβ - transforming growth factor beta

Th1 - T helper lymphocyte type 1

Th2 - T helper lymphocyte type 2

TLR4 - Toll-like receptor 4

TNFα - tumor necrosis factor alpha

TNFR1 – tumor necrosis alpha receptor type 1

TNFR2 – tumor necrosis alpha receptor type 2

TRPA1 - transient receptor potential ankyrin 1

TRPV1 - transient receptor vanilloid type 1

TZD – thiazolidinedione

VEH - vehicle

WDR - wide-dynamic range neurons

15d-PGJ2 - 15-deoxy Δ12,14 prostaglandin J2

1. INTRODUCTION

Pain is a principal integral part of the body's protective system. The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Acute sensation of pain results in reflexive retraction from the painful stimulus, and tendency to protect the affected body part while it heals, as well as to avoid painful situation in the future. The IASP definition of pain also suggests that pain is a highly subjective matter, as the equal stimuli may induce various reactions in different individuals. Indeed, the intensity of pain perception depends on sex, age, psychological state or even mood.

However, under pathological conditions pain loses its protective function and becomes a problem itself. Pathological **neuropathic pain** represents a main sign of **neuropathy**, a highly debilitating disorder that has a robust impact on the quality of individual's life. The variety of causes that trigger pathological changes in pain processing has been studied from very beginning of human medicine, as pain is the main symptom of organism's dysfunction at many levels. Neuropathic pain may be induced by injury of peripheral or central nervous system by various mechanisms involving traumatic injury, metabolic damage, chemotherapy and virus infection. The changes in **nociception** (the detection, transduction and transmission of nocuous stimuli to specialized brain structures) and/or in **pain perception** may be influenced also by stress, anxiety or different psychiatric disorders. Despite of long lasting extensive study, the mechanisms underlying neuropathic pain development are still not understood completely, hence, treatment strategies are not satisfactory for now.

Inflammatory changes in the nervous system, also referred as **neuroinflammation**, usually accompany the development and maintenance of neuropathic pain. The intensity and extent of the neuroinflammatory reaction is highly dependent on the type and severity of injury to the nervous system. Additionally, different neuroinflammatory changes take place in central and peripheral nervous systems. Spinal cord is considered as immune-privileged tissue, as it is protected with blood-spinal cord barrier (BSCB) and is equipped with immune-like glial cells - microglia. Microglia, in cooperation with astrocytes, become activated in reaction to specific stimuli, produce pro-inflammatory factors and subsequently modulate nociceptive signal transmission.

Anticancer paclitaxel treatment often induces unwanted side effect called paclitaxel-induced peripheral neuropathy (PINP), which is a common cause for treatment

termination. The exact mechanisms underlying this phenomenon remain unclear, causing a significant therapeutical challenge. Recent studies suggest the neuroinflammatory changes in both peripheral and central levels to play a pivotal role in the establishment and maintenance of peripheral neuropathy caused by chemotherapy and peripheral injury as well. Losartan is a widely used Angiotensin II receptor type 1 (AT1R) blocker. Besides its antihypertensive effect, it was also studied for anti-inflammatory and neuroprotective properties on different models of neurodegenerative diseases. Systemic losartan treatment attenuated both neuropathic pain-like behavioral changes and the expression of pro-inflammatory proteins induced by spinal nerve injury (SNL) or PAC administration (Publications A (Kalynovska et al., 2019) and B (submitted manuscript)). Moreover, losartan treatment induced the expression of pro-resolving markers in dorsal root ganglia (DRGs) and spinal cord dorsal horns (SCDH) of PAC-treated rats, suggesting immune-like cells as its primary target. Modulation of neuroimmune response to different triggers represents here an interesting and promising target for elaboration of novel anti-neuropathic treatment strategies.

On the central level, the incubation of spinal cord slices with chemotherapeutic paclitaxel (PAC) led to the neuronal activation in the superficial SCDH. The underlying mechanism of PAC action involve transient receptor vanilloid type 1 (TRPV1) activation, as TRPV1 antagonist was able to abolish the paclitaxel-induced neuronal activation (Publication C (Kalynovska et al., 2017)).

Chemokine C-C motif ligand 2 (CCL2), produced mainly by astrocytes, but also released from the central terminals of nociceptive neurons under pathological conditions, was shown to mediate central sensitization of spinal cord dorsal horn neurons and to induce painful reaction to otherwise innocuous mechanical stimuli (Publication D (Spicarova et al., 2014a)). This mechanism is also revealed to be TRPV1-dependent, as TRPV1 antagonist was able to prevent from CCL2-induced behavioral, molecular and electrophysiological changes. This doctoral thesis is primarily focused on the study of neuroinflammatory changes during chemotherapy- and injury-induced peripheral neuropathy states. Another part of this thesis is devoted to the mechanisms of central sensitization at the spinal cord level in nociceptive signaling. Considering the diversity of possible pathological triggers, it is clear that mechanism-based treatment strategies should be essential in the elaboration of potentially safer and more effective medication for peripheral neuropathy.

2. STATE OF THE ART

2.1 Anatomy and physiology of pain

The Kyoto protocol of the International Association for the Study of Pain (IASP) Basic Pain Terminology clearly defined the term **nociception** as "the neural processes of encoding and processing noxious stimuli" (Jung et al., 2008). It is important to emphasize that pain is a subjective experience while nociception is a physiological sensory process. Nociception consists of signal transduction by nociceptors in peripheral nerve endings, signal transmission via primary afferent fibers (PAF) to the second-order neurons in the spinal cord dorsal horn (SCDH), and through ascending pathways to brainstem reticular formation, thalamus, limbic system and somatosensory cortex. Thalamus is mostly responsible for physiological reactions to pain stimuli; somatosensory cortex covers topographical representation and limbic system for negative experience and memory.

2.1.1 Transduction and transmission

Transduction is a process of converting stimuli, such as pressure, thermal energy, or chemical irritation, into a nerve signal (e.g., an action potential). Pain transduction takes place at the peripheral endings of primary afferent neurons whose terminals (nociceptors) are sensitive to this particular type of activation. The specificity of nociceptors was confirmed by electrophysiological studies (Burgess and Perl, 1967), when the selective sensitivity of nociceptors to noxious, but not to innocuous, stimuli was detected. Different sensory modalities are defined by the expression of multiple sensory channels and receptors on the terminal branches of nociceptors that allow them to respond to the specific noxious stimuli (Liu and Ma, 2011). Nociceptors differ from other sensory neurons not only with high specificity to stimulus, but also with increased activation threshold. The sensory information is subsequently encoded into trains of action potentials and is further transmitted from the periphery to the central nervous system (CNS) via primary sensory neurons. Primary sensory neurons, also called primary afferent fibers (PAF), are pseudounipolar neurons with their cell bodies in the dorsal root ganglia (DRGs). Two types of primary sensory neurons are known - unimodal and polymodal, depending on their capacity to respond to one or more types of stimuli (Julius and Basbaum, 2001).

Under normal conditions, two types of PAF are involved in nociception - Aδ- and C- fibers. Aδ are myelinated medium-sized fibers (2-6 μm) with intermediate velocity of 12-30 m sec^{-1} are responsible for the experience of sharp pain. C-fibers are unmyelinated thin fibers (0,4-1,2 μm in diameter) with small cell bodies and slow conduction(0,5-2,0 m sec-1), are usually associated with delayed and blunt pain (Millan, 1999) (Figure 2.1). C-fibers are also polymodal, hence are able to respond to noxious thermal, mechanical and chemical (e.g. capsaicin) stimuli and inflammatory mediators (Nagy and Rang, 1999).

2.1.2 Neuronal cell types in DRG

Primary afferent nociceptors belong to pseudounipolar neurons with cell bodies situated in DRG. They give off a single process, which is divided into peripheral and central branches. Peripheral terminals innervate a target organ and central branch passes into the spinal cord and forms synapse with second-order neurons mainly in the superficial laminae. DRG neurons are classified by their size and function. Cells with the largest diameters give rise to rapidly conducting, myelinated Aβ- fibers. Usually, these fibers are not involved in nociceptive transmission, however, under pathological conditions, their activation may contribute to painful sensations (Julius and Basbaum, 2001). Medium- and small-diameter neurons give rise to Aδ- and C-fibers respectively (Liu and Ma, 2011). According to the expression of molecular markers, C-nociceptors are subdivided into peptidergic and nonpeptidergic populations. Peptidergic C-fibers express calcitonin-gene related peptide (CGRP) and substance P and are capable to respond to nerve growth factor (NGF). Another characteristic feature of peptidergic neurons is expression of TRPV1 and tetrodotoxin-resistant voltage-gated sodium channel type 1.9 (Nav1.9) (Fang et al., 2002). The nonpeptidergic neurons bind the plant isolectin B4 and express the c-Ret neurotrophin receptor, which is activated by glial-derived neurotrophic factor (GDNF) (Basbaum et al., 2009).

2.1.3 The spinal nociceptive network

The spinal cord is so-called „gateway“ for the relay of nociceptive signaling from the periphery to the CNS. According to cytoarchitectonic characteristics, spinal cord neurons are divided into 10 parallel layers called laminae (Rexed, 1952). Peptidergic C-fibers terminate in the most superficial lamina I (marginal zone) and the dorsal part of lamina II (*substantia gelatinosa*), nonpeptidergic afferents project to mid-region of lamina

II, while high threshold Aδ-fibers terminate both in laminae I and II. Notably, both types of nociceptors provide also an input to laminae V and VI, however, to a lesser extent (Basbaum et al., 2009). Larger, low threshold, Aβ fibers selectively innervate deeper dorsal horn laminae (III-IV, less markedly in laminae V/VI) or ascend to brainstem dorsal column nuclei through dorsal column-medial lemniscus pathway immediately after entering the spinal cord (Figure 1.1.)(Berger et al., 2011).

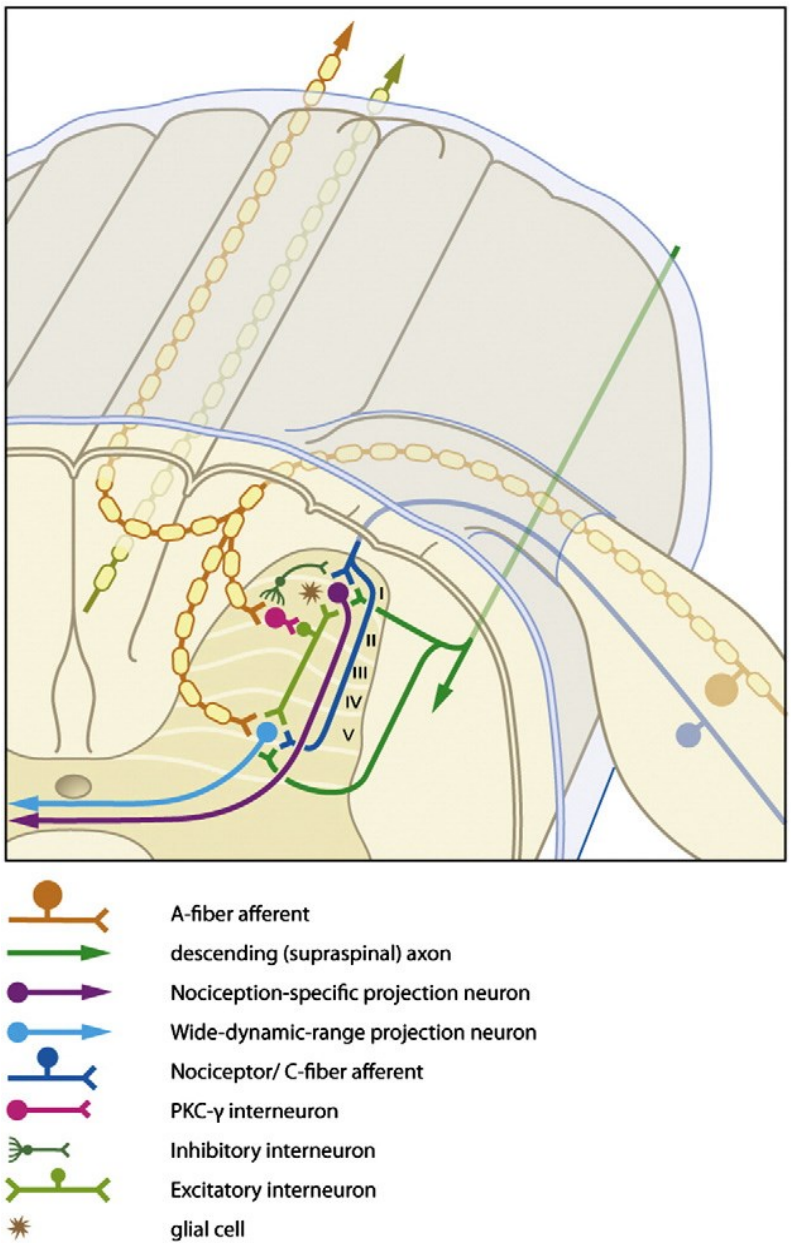

Figure 1.1. The spinal nociceptive network. *Adapted from (Berger et al., 2011).*

Based on the activity response type, three types of projection neurons in the SCDH may be identified: low threshold (LT), high threshold (HT) and wide-dynamic range

(WDR). LT neurons respond to innocuous stimulation from Aβ-fibers, while HT are nociceptive-specific neurons which are activated exclusively by high intensity, noxious stimuli mediated by C and Aδ-fibers. HT neurons are mostly concentrated in the superficial dorsal horn. WDR neurons may be excited by thermal, mechanical and chemical stimuli mediated via C and Aδ, as well as Aβ-fibers (Millan, 1999, Berger et al., 2011). Lamina II contains also the largest population of interneurons. According to the main neurotransmitter released, interneurons are divided into excitatory (those producing glutamate) and inhibitory (glycinergic and gamma-aminobutyric acid (GABA)-ergic interneurons). Two inhibitory systems in the spinal nociceptive network has been described – segmental (glycinergic and GABAergic interneurons) and non-segmental. The non-segmental inhibitory system consists of descending (aminergic) fibers from the higher centers of CNS (Saade and Jabbur, 2008).

Following the nociceptive signal transmission from peripheral afferent neurons to projection neurons in SCDH, it is subsequently carried out via ascending pathways to the higher brain centers. Ascending nociceptive pathways are commonly divided into two types - monosynaptic (anterolateral system, ALS) and polysynaptic pathways. One of the most important for nociception is monosynaptic pathway the spinotalamic tract (STT), which transmits integrated nociceptive signals to ventral posterolateral and posteromedial nuclei in the thalamus, where sensory information about the location and intensity of the noxious stimulus is relayed to the somatosensory cortex. Spinoreticular tract leads signal to reticular formation, and spinomesencephalic tract ends in periaqueductal grey. Polysynaptic pathways include dorsal column-medial lemniscus pathway and spinocervical tract, however they do not participate nociceptive transmission directly. Sensory information from face and head is processed by trigeminal nerve.

2.1.4 The supraspinal processing of nociceptive information

The majority of nociceptive information is projected to thalamus via contralateral STT, medulla and brainstem via spinoreticular and spinomesencephalic tracts. Thalamus is considered as main relay site for nociceptive inputs to somatosensory cortex, essential for pain stimuli discrimination and complex planning of affective response. Brainstem structures are involved in the integration of nociceptive activity with homeostatic and autonomic processes (Tracey and Mantyh, 2007). The cingulate and insular cortices receive information via connections with the brainstem (parabrachial nucleus) and

amygdala, and maintain the emotional aspect of the pain sensation. Other important structures of the brain nociceptive system are the rostral ventral medulla (RVM) and periaqueductal gray (PAG). These structures located in the midbrain, control the descending feedback systems, which may induce the facilitation (pro-nociceptive) or inhibition (anti-nociceptive effect) of nociceptive signal processing mainly in SCDH. The circuit that includes PAG, the locus coeruleus and the nucleus raphe magnus, makes the largest contribution to antinociceptive descending modulation.

2.2 Non-neuronal cells in central and peripheral nervous systems

Central and peripheral nervous system (PNS) are the most complex and sophisticated systems in organism. They are formed by cells of numerous types, functions and origin, which generally may be subdivided into neuronal and non-neuronal (glial) cells.

2.2.1 Glial cells

Until recently, glial cells were considered mostly as just supporting cells for neurons. However, it is now clear that glia, representing the majority of all cells in adult nervous system, performs essential functions - from guiding developing neurons to their destination sites to mediation of the apoptosis and degradation of old or damaged neurons. Three main types of glial cells - astrocytes, oligodendrocytes and microglia- fulfill all those functions mentioned above (Figure 1.2.).

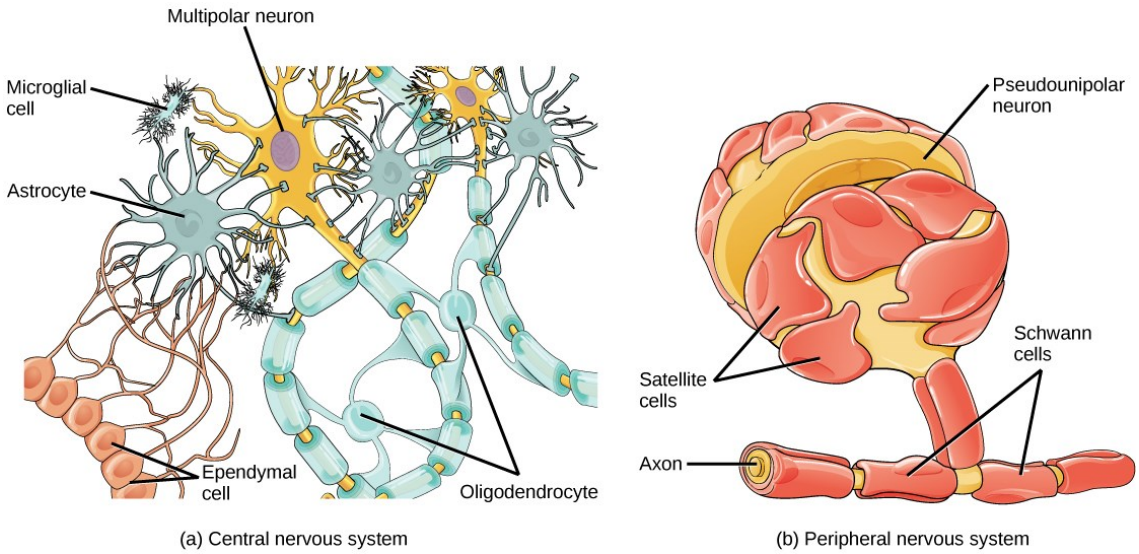

Figure 1.2. Interactions between neurons and glial cells in the central (a) and peripheral (b) nervous system. *Ilustration is adapted from OpenStax Collage. Neurons and Glial Cells. Biology.*

Astrocytes are the most abundant glial cells in the CNS, named for their star-like shape. These cells form contacts with blood vessels and neurons and maintain neuronal energy metabolism via circulating glucose uptake, metabolizing it to lactate, which is further delivered to neurons to fulfill their metabolic demands (Jha and Morrison, 2018). Astrocytes also mediate neurotransmitter clearance (via glutamate and GABA uptake) and dynamically control extracellular K^+ concentrations, thus maintaining normal neuronal excitability. A wide range of expressed receptors enables them to register and respond to different neurotransmitters and other active substances in extracellular fluid (ECF) by generating intracellular Ca^{2+} signals, which are spreading via gap-junctions to neighboring astrocytes. This signaling cascade often leads to proper physiological feedback including the release of so-called gliotransmitters: glutamate, GABA, purines, cytokines and different peptides or metabolites (Min et al., 2012). Astrocytes are also called glue in CNS as they form a matrix that keep neurons in proper spatial position and form neural scars when the tissue is regenerating after the injury. They also play a prominent role in establishing and functioning of the blood-brain barrier (BBB), which will be described in detail in the next chapter. **Satellite glial cells** surround sensory neurons in DRGs, providing nutrition and structural support, and are thought to be an equivalent of astrocytes in the CNS.

The main function of **oligodendrocytes** is to form myelin lipid bilayers, which insulate axons. One oligodendrocyte can provide the myelination for several neurons, as well as one neuron may be myelinated by few oligodendrocytes. In the PNS, every

Schwann cells insulate only one axon as an entire cell forms single myelin segment. Recently, satellite oligodendrocytes were identified, which are functionally different from the rest oligodendrocytes. Along with the providing myelin sheaths, they help astroglia regulate the extracellular fluid and glutamate homeostasis, thus influencing the activity of surrounding neurons (van Landeghem et al., 2007).

Microglia represent an essential form of immune defense in the CNS and have no analogues in the periphery. Unlike the other glial cells that originate from neuronal stem cell lineage, microglia arise from erythro-myeloid precursors in the yolk sac and migrates to the CNS already during early embryonic development. While microglia has common precursors with monocytes (type of white blood cells mediating inflammatory response), it differs with constant self-renewal strategy within the same site (brain or spinal cord) instead of renewal from the circulating bone marrow hematopoietic stem cells (Liu et al., 2010). In so-called "resting" state, microglia represents small, ramified cells involved in the structural plasticity through the release of cytokines and growth factors (Morris et al., 2013). Microglia is known for its high activation capacity. Inflammation or nerve injury promotes microglia cells to activation state, during which it changes the shape to amoeboid, migrate to the signaling site and perform cytotoxic, phagocytic and antigen-presenting functions. Activation processes will be described in detail in the chapter *Neuroinflammatory changes in the CNS.*

The less abundant type of CNS non-neuronal cells is **ependymal cells**. They line fluid-filled ventricular system in brain and the central canal of the spinal cord. Ependyma is neuroepithelial layer mainly involved in the production and regulation of cerebrospinal fluid (CSF).

2.2.2 Blood-brain barrier and its analogues

Human brain consumes 20% of body glucose and oxygen; hence, it is highly vascularized in order to fulfill its metabolic and energy demands. Specific dynamic structure called blood-brain barrier (BBB) protects brain neurons from infections or injuries and maintains CNS microenvironment and homeostasis. BBB separates CNS from circulation with complex structure formed by nonfenestrated endothelial cells, pericytes and astrocyte foot processes. BBB is not a rigid structure as it was first assumed; it is plastic and adaptive in response to various factors. A wide diversity of ion channels, pumps and transporters expressed on BBB membranes enables highly selective exchange between circulation and parenchyma. From immunological point of view, BBB is not just a

barrier of first line defense, but rather is an active contributor to the innate immune response in the CNS mostly by modifying its permeability in reaction to specific signals (Lampron et al., 2013).

In the spinal cord, blood-spinal cord barrier (BSCB) is considered as BBB equivalent. Although they share similar building structures and morphological features, BSCB is generally more permeable to several cytokines including interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), indicating higher probability of BSCB disruption or malfunction during pathological states (Bartanusz et al., 2011).

Another analogue of BBB in the PNS is blood-nerve barrier (BNB), consisting of endoneurial microvessels within the nerve fascicle and the investing perineurium. It plays an important role in nerve development, Wallerian degeneration and peripheral sensitization contributing to neuropathic pain syndrome (Weerasuriya and Mizisin, 2011).

While the brain, spinal cord, and peripheral nerves are protected with respective barriers, the DRGs lack any diffusion barrier (Liu et al., 2018). This morphological issue makes the DRGs susceptible to different circulating molecules that have limited access to the abovementioned immune privileged organs.

2.3 Classification of pain states

The essential function of nociceptive pain is to evoke complex withdrawal response in order to interrupt and to minimize the contact with noxious stimuli, in this way protecting body from potential damage. According to its duration, pain is usually classified to acute, subchronic and chronic pain. **Acute pain** is normal physiological reaction to noxious stimuli, which directly activate the nociceptors in the skin (cutaneous pain) or in deep viscera (visceral pain). It lasts only upon the presence of its trigger and disappears after the stimulus is removed. **Subchronic pain** lasts for few hours up to days and fades away when the normal body integrity is restored. If pain persists for three to six months, it is classified as **chronic pain**. In this case, it is usually present without any obvious cause, or manifested as response of inadequate magnitude due to its trigger (Millan, 1999). Subchronic and chronic pain states are characterized by spontaneous pain, increased response to noxious stimuli (hyperalgesia) or painful reactions to normally innocuous stimuli (allodynia)(Figure 1.3.).

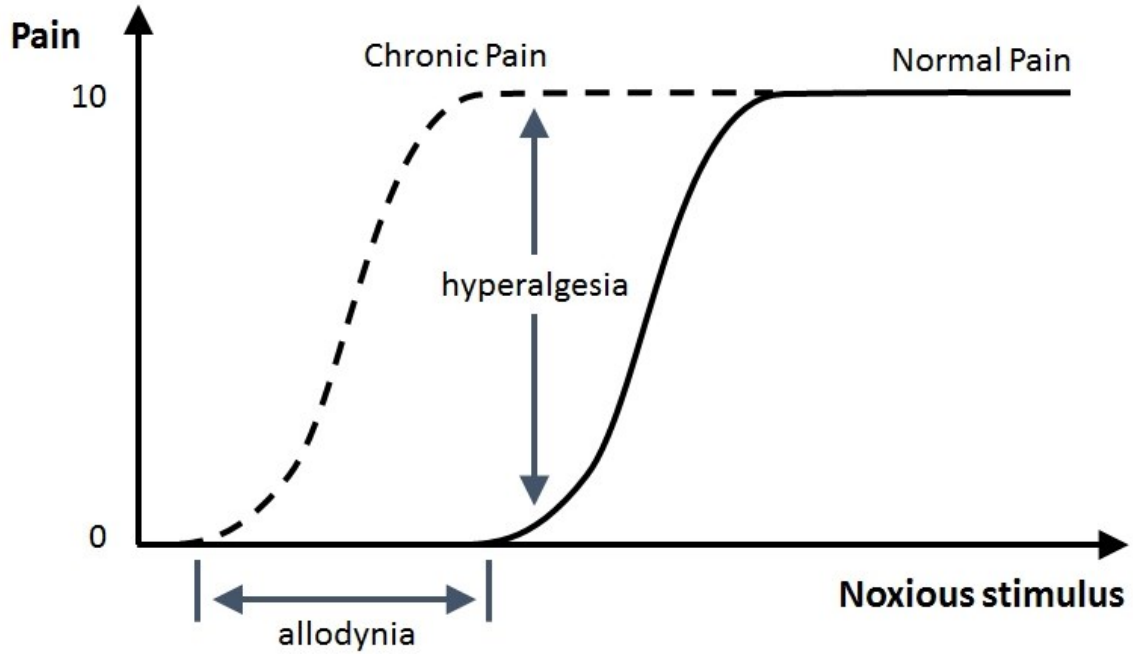

Figure 1.3. Schematic illustration of altered pain perception phenomena during chronic pain states. *Adapted and modified from activelifescientific.org.*

In contrast to the nociceptive pain triggered by tissue damage, **neuropathic pain** results from lesion or disease of the nervous somatosensory system. It is important to emphasize that neuropathic pain is not equal to the chronic pain. Regarding inflammatory pain, when pain sensation is triggered by the activation of nociceptors with chemical mediators at the site of inflammation (rheumatoid arthritis), chronic pain states may occur, but it cannot be characterized as neuropathic as neural tissue remain intact and functional (Costigan et al., 2009). Understanding the differences between these types of pain is essential in determination of treatment strategies, as the mechanisms involved in their development are distinct.

2.4 Neuropathic pain

Neuropathic pain is officially defined as a pain caused by lesion or disease of somatosensory system (Woolf, 2010). While nociceptive pain is helpful in indication of damaging or potentially dangerous factors, neuropathic pain is a signal of malfunctions in

registration, processing or perception of pain. Common conditions associated with neuropathic pain may be divided to several groups according to the type of damage that triggered neuropathy (Zimmermann, 2001):

- mechanical nerve injury (carpal tunnel syndrome, vertebral disk herniation);
- metabolic disease (diabetic polyneuropathy);
- viral disease (herpes zoster, HIV);
- neurotoxicity (chemotherapy-induced peripheral neuropathy - CIPN);
- neural focal ischemia (anesthesia dolorosa);
- neuroinflammation (multiple sclerosis);
- multiple neurotransmitter system dysfunction (complex regional pain syndrome).

Research in the pain field has focused on the study of underlying pathological mechanisms of neuropathic pain using well-established rodent models. These models mimic human clinical conditions and are generally based on the injury of CNS or PNS, local or systemic administration of drugs, or *in vitro* techniques.

2.4.1 Pathophysiology of neuropathic pain

Neuropathic pain represents an unsolved challenge in clinical practice, as current treatments are still unsatisfactory. Current treatment strategies for neuropathic pain include mostly powerful anesthetics, e.g. opioids or selective channel blockers as gabapentin; also anticonvulsants or antidepressants, which have limited positive effect while side effects are more pronounced (Attal et al., 2009). Traditional treatment procedures as neuronal blockade, steroid injections, stimulations of spinal cord, DRGs and peripheral nerves; epidural or transcranial cortical stimulation along with following physical and psychological therapies, have a huge impact on the quality of patients' life. Modern research is focused on the identification of novel therapeutic targets for elaboration of mechanism-based treatment approaches, which are considered to be more effective.

Generally, neuropathic pain is a symptom of either central neuropathy if neuroplastic changes occur in brain or spinal cord, or peripheral neuropathy, characterized by lesions in PNS, namely peripheral afferent terminals. Undoubtedly,

malfunctions on any level of somatosensory system result in alterations of pain perception - hyperalgesia and/or allodynia.

2.4.2 Changes in nociceptive signaling

Neuropathic states are primarily characterized by the alterations in excitation and/or transduction properties of sensory nerves on the periphery, accompanied with malfunctions in the central nociceptive network, such as synaptic plasticity in the SCDH, impairment of spinal excitatory/inhibitory circuits and descending pain control system as well. These alterations lead to the development of the hyperexcitability of nociceptive system, which is also called **sensitization**. It is clear, that potentiation of excitability is based on the changes in ion channels (sodium, calcium and potassium) functions. **Peripheral sensitization** is usually accompained with the ectopic activity of sensory nerves, when action potentials are generated by proximal parts of injured nerve fibers as a result of spontaneous oscillations in their membrane activity (Liu et al., 2000). Additionally, phenotypic switching and structural plasticity of peripheral afferent fibers including sympathetic nerve sprouting in DRGs are also common for peripheral neuropathy states (Weissner et al., 2006, Berger et al., 2011). Nearby putative intact fibers also undergo changes and become hyperexcitable (Bernal and Roza, 2018). Moreover, neuropathic pain conditions trigger an enhanced expression of $\alpha_2\delta$ subunits of calcium channels by small diameter neurons in affected DRGs (Patel and Dickenson, 2016). These subunits are crucial in retrograde transport of N-type calcium channels to the central terminals, where they presynaptically control calcium influx and glutamate release, inducing subsequent synaptic plasticity (Hoppa et al., 2012). Abovementioned alterations on the periphery may result in enhanced excitatory synaptic transmission in the SCDH, called **central sensitization**.

Central sensitization plays a key role in the development and maintenance of the neuropathic pain states. This abnormal enhancement in functions of nociceptive neurons and circuits in the SCDH is characterized by an increase in membrane excitability, synaptic efficacy, and by the attenuation of inhibition as well. These changes in electrical properties of neurons result in reduction of activation threshold, which in turn leads to the increased responsiveness to peripheral stimulation (Latremoliere and Woolf, 2009).

Enhanced peripheral stimulation leads also to the postsynaptic changes in second-order nociceptive neurons due to a concomitant release of excitatory neurotransmitters and neuropeptides. Being the principal neurotransmitter in the SCDH, **glutamate** is excessively released into the synaptic cleft in a result of ongoing discharge

from PAFs. These alterations results in plastic changes on the postsynaptic membrane, such as the increased expression and phosphorylation of glutamate receptors (α-amino-3 hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors (Woolf and Salter, 2000). Activity-depending **synaptic plasticity** leads to the facilitation of nociceptive signal input into the highest CNS centers, resulting in pain hypersensitivity. Increased nociceptive input from PAFs includes also the release of neuroactive substances in the SCDH, such as substance P, CGRP, brain-derived neurotrophic factor (BDNF) or bradykinin. After the binding to the specific receptors, these substances may affect intracellular signaling pathways, and thus contribute to the synaptic plasticity in the SCDH. It is known that bradykinin or CGRP are able to activate protein kinase A (PKA), which in turn phosphorylates NR1 subunit of NMDA receptor (Latremoliere and Woolf, 2009). It was shown by Chen and colleagues that BDNF activates also presynaptic NMDA receptors through Tropomyosin receptor kinase B-dependent mechanism, contributing to increased excitability in the SCDH (Chen et al., 2014b).

Central sensitization is an imbalance between excitatory and inhibitory mechanisms in central nociceptive circuits. Besides the facilitation of the ascending nociceptive signaling, neuropathic pain states are also characterized by impairment of inhibitory mechanisms in the SCDH, called **disinhibition**. It may be manifested as a local loss of inhibitory neurons as a result of NMDA receptors-dependent glutamate excitotoxicity (Scholz et al., 2005), reduction of segmental inhibition (Coull et al., 2005) or decreased descending endogenous inhibition. Reduced inhibitory input from the supraspinal structures leads to the decrease in activity of spinal α2 adrenergic or $5HT_7$ serotonergic receptors, thus facilitating nociceptive transmission and contributing to neuropathic pain development (Ossipov et al., 2010).

Structural changes in the spinal cord dorsal horn may also contribute to the development of abnormal pain sensations. As it was described above, Aδ and C-fiber terminals carry nociceptive information and terminate in laminae I and II, while Aβ convey innocuous tactile information and synapse primarily onto laminae III and IV. Injury of peripheral nerves can result in degeneration of DRG cells and loss of C-fiber terminals in superficial SCDH (Arvidsson et al., 1986). It was suggested that compromised inhibitory modulation and tissue regenerative response trigger nerve sprouting of Aβ-fibers from the deeper laminae to the superficial area. This process represents one of the possible mechanisms of allodynia generation, when touch is processed as a painful signal (Alles and Smith, 2018).

Alterations in supraspinal processing of nociceptive information also contribute to central sensitization. Projections from forebrain areas such as cingulate cortex and

amygdala modulate descending inhibitory signals from PAG, facilitating disinhibition in SCDH. Moreover, enhanced descending facilitatory system, specific activation of limbic and paralimbic circuits along with increased activation of prefrontal cortex and brainstem alters pain processing and perception and contributes to the development of neuropathy (Tracey and Mantyh, 2007).

2.4.3 Neuronal activation in the SCDH

The activation of peripheral afferents by various noxious stimuli also leads to a rapid and mostly transient induction of early-gene expression and/or phosphorylation of signaling molecules in spinal nociceptive neurons (Gao and Ji, 2009). Already thirty years ago, Hunt and colleagues reported robust induction of **c-Fos** protein expression in spinal cord superficial laminae following peripheral noxious stimulation (Hunt et al., 1987). c-Fos is a protein product of early gene *c-fos*, which is expressed in the cell perinuclear cytoplasm of the SCDH neurons, hence, it is widely used as a marker of nociceptive neuron activation upon noxious peripheral stimulation. c-Fos expression may be induced by various stimuli, including thermal, mechanical, chemical and electrical as well (Gao and Ji, 2009). Notably, the topographical correlation of c-Fos-positive neurons and central terminals of peripheral afferents in the superficial area of the SCDH, as well as stimulus-dependent c-Fos expression pattern, strongly suggest the involvement of c-Fos signalization in nociception and neuronal plasticity (Palecek et al., 2003, Uchytilova et al., 2015). *c-fos* gene has relatively low basal expression and is rapidly (within minutes up to 30 minutes) induced by noxious peripheral stimulation (Draisci and Iadarola, 1989). After approximately 1 hour delayed translation into protein, the last is translocated to the nucleus, where forms homo- or heterodimers with other nuclear proteins, resulting in sequence-specific DNA-binding transcription factors of activator protein 1 (AP-1) family (Vesely et al., 2009). AP-1 transcription factors are known to be implicated in the regulation of cell proliferation and differentiation, growth and apoptosis. Hence, c-Fos represents a link between extracellular events and reciprocal cell response.

Along with the induction of c-Fos expression, noxious peripheral stimulation also activates the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) in superficial SCDH, the area where peripheral afferents terminate (Ji et al., 1999). Phosphorylated ERK (**pERK**) is also an often used and well-established marker of cell activation by noxious stimuli. Like c-Fos, pERK expression is induced by the variety of stimuli, and is intensity-dependent (Gao and Ji, 2009). However, there are some

discrepancies. First, while c-Fos is located in neuronal nuclei, pERK also occurs in cytoplasma, axons and dendrites of affected neurons. Moreover, pERK was demonstrated in spinal microglia and astrocytes under different pathological conditions (Ma and Quirion, 2005, Wang et al., 2011). Second, ERK phosphorylation after high-threshold stimulation is relatively faster, when compared to c-Fos expression. pERK is elevated already within a minute with a peak in 2-5 minutes after stimulus application, and returns to basal level after 2 hours (Ji et al., 1999). When phosphorylated, ERK is translocated to the nucleus, where sequentially activate transcriptional factors involved in cell mitosis, proliferation and differentiation. Given the active role of c-Fos and pERK in dynamic cell response to noxious peripheral stimulation, it is clear that these activation markers play a substantial role in synaptic plasticity and central sensitization during neuropathic pain states.

2.4.4 The role of TRPV1 in the development of neuropathic pain

The indisputable role of TRPV1 receptors in nociceptive modulation has been intensively studied from their identification in 1997 (Caterina et al., 1997). TRPV1 receptors are polymodal non-selective cation channels, formed by four subunits consisting of six transmembrane domains with a hydrophobic pore-forming stretch between fifth and sixth domain. TRPV1 receptors are also called "capsaicin receptors" as the pungent compound of hot chili peppers – vanilloid capsaicin, activates them. Additionally, they are also activated by various stimuli, such as noxious heat (>43°C), protons (pH<5), voltage, secondary messengers, cytoskeleton and various endogenous lipids (Cui et al., 2016). Functional properties of TRPV1 receptors may be also modulated via phosphorylation by protein kinase C (PKC), protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). TRPV1 receptors are expressed in nociceptive small-diameter DRG neurons, their central and peripheral terminals, and also in several brain structures (Edwards, 2014). Due to the activation by a broad array of noxious stimuli, TRPV1 receptors have been suggested as a principal molecular integrator for different types of nociceptive input. Hence, their role in the detection of noxious stimuli and modulation of pain transduction in the periphery has been actively studied and now is well-established (Nagy et al., 2014). However, the role of TRPV1 receptors expressed in the central terminals is not completely understood (Spicarova and Palecek, 2009, Spicarova et al., 2014b). Considering their location (presynaptic terminals in the superficial SCDH) and functional versatility, it is clear that they play pivotal role in modulation of nociceptive

transmission, contributing to synaptic plasticity, and thus to the central sensitization. Their implication has been described for different pathological conditions, including chemotherapy-induced peripheral neuropathy (Hara et al., 2013, Li et al., 2015), peripheral injury-induced neuropathic pain and neuroinflammation (Matta and Ahern, 2011, Van Steenwinckel et al., 2011, Spicarova et al., 2014a). TRPV1 receptors are considered as a key player in pathologic pain establishment, thus represent primary target in innovative neuropathic pain management strategies.

2.5 Neuroinflammation

Growing number of studies suggest that inflammation of neural tissue represents a key mechanism of neuropathic pain development after the nerve injury. It is now admitted that damage or injury to neural structures, as well as metabolic changes and chemical irritation, initiate the inflammatory response of the neural tissue – **neuroinflammation**. It is a highly complex process that involves neuronal, glial and/or immune cells, which undergo activation and cause pathological alterations in nociceptive signaling (Ellis and Bennett, 2013). Depending on the type of the injury, neuroinflammatory changes are manifested both in peripheral and central nervous systems to a different extend. The pathogenesis of neuroinflammation is based on the activation of inflammatory and immune-like glial cells, for example satellite glial cells, mast cells, neutrophils, macrophages and T-cells at the peripheral level and microglia and astrocytes in the CNS (Moalem and Tracey, 2006). At the site of the injury, neuroimmune response may provoke an increase in local perfusion, capillary permeability and concentration of pro-inflammatory mediators, causing further activation of innate immune response. As any inflammatory response, neuroinflammation consists of two main phases – pro-inflammatory, when the pathogen removal and debris cleaning takes place, and pro-resolving phase, during which the immune reaction is primarily oriented on the local tissue repair and common homeostasis reestablishment (Ellis and Bennett, 2013). Both phases are mediated by different cell types or subtypes and involve distinct mechanisms, conditioned by the localization and magnitude of neuroinflammatory response (Figure 1.4).

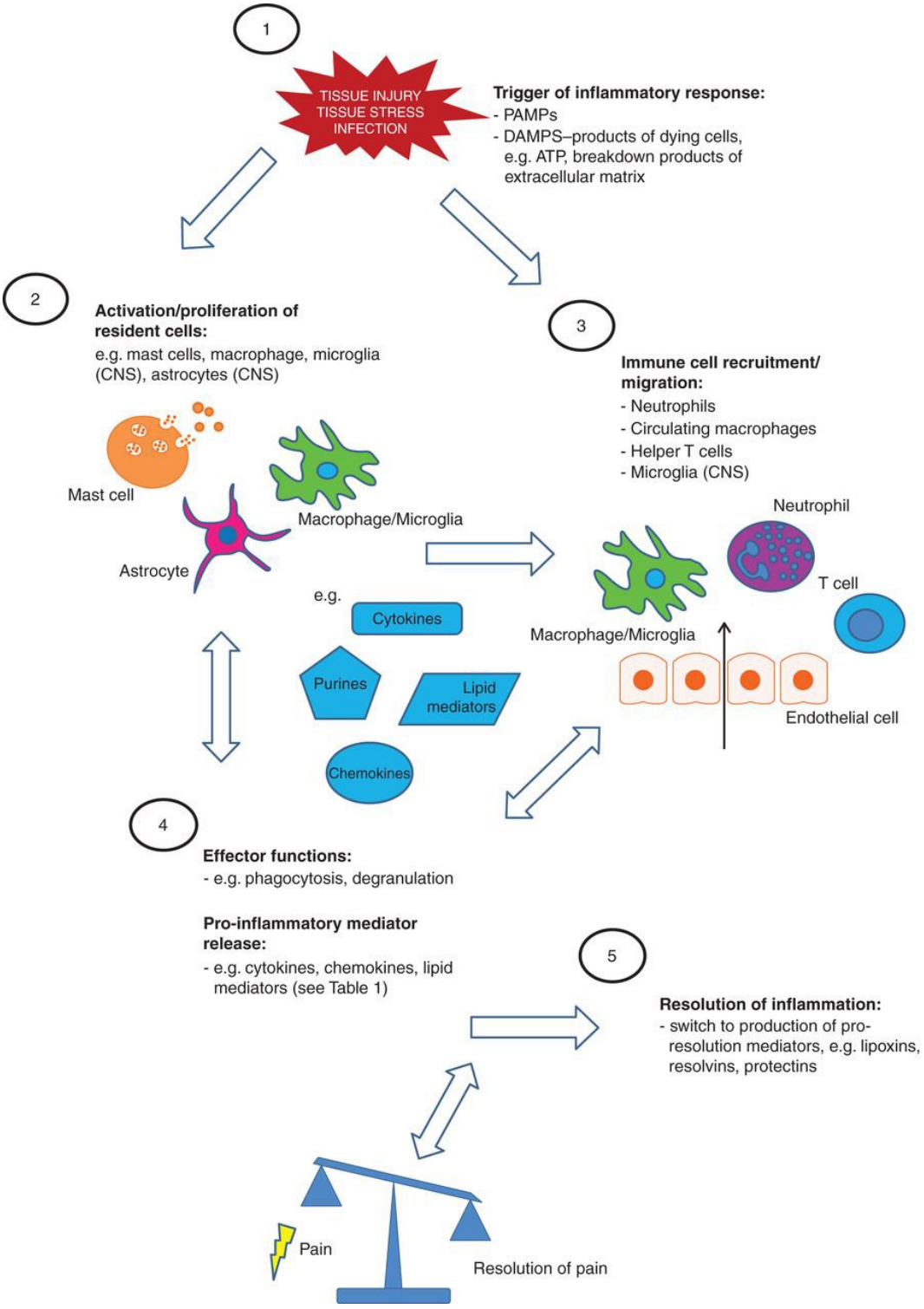

Figure 1.4. Schematic illustration of common neuroimmune response. *Adapted from (Ellis and Bennett, 2013).*

2.5.1 Neuroinflammation in the periphery

Peripheral nerve injury leads to immediate local inflammatory response orchestrated predominantly by glial and resident immune-like cells. While the exact activation mechanism is still not completely clear, it is generally believed that Schwann cells, resident macrophages and neutrophils are the first cells to react to nerve damage. Myelinating Schwann cells immediately react to the damage or injury of nerve axon via ERK mitogen-activated protein kinase (MAP) kinase signaling pathway (Napoli et al., 2012). Increased concentration of adenosine and bradykinin at the site of the injury triggers the activation of resident mast cells (Moalem and Tracey, 2006). Once activated, resident mast cells undergo degranulation, which leads to the release of histamine, serotonin, nerve growth factor, TNF-α, and other mediators. These pro-inflammatory mediators are known to sensitize nociceptors, as well as to promote the infiltration of circulating immune cells such as neutrophils and monocytes (Perkins and Tracey, 2000, Zuo et al., 2003). Neutrophils, polymorphonuclear leukocytes, extravasate from small blood vessels to the site of the injury and contribute to the local inflammation. Recruited monocytes infiltrate to the site of the injury and then differentiate into macrophages, which together with resident macrophages and Schwann cells provide phagocytosis and degradation of damaged axons and myelin sheaths. Moreover, invaded macrophages orchestrate local immune response via release of cytokines, chemokines and other pro-inflammatory mediators (Nathan, 1987). At the injury site, macrophages represent the largest population among invaded cells, indicating their key role in the regulation of neuroinflammation (Kiguchi et al., 2017).

The variety of receptors expressed in macrophages enables their rapid activation by the chemotactic molecules in microenvironment, namely by chemokines. Chemokines are small secreted molecules, named for their leukocyte chemoattractant and cytokine-like properties (Asensio and Campbell, 1999). Chemokine family consists of few subfamilies – CC, CXC, XC, and CX3C. The CC subfamily is the largest among them and is particularly important for study of neuropathic pain development, as number of studies indicated their role not only in chemotaxis, but also in direct modulation of nociceptive transmission. Increased expression of CCL2, also referred as monocytes chemoattractant protein 1 (MCP-1), is well documented in DRG neurons in different models of peripheral nerve injury, including partial ligation of the sciatic nerve (Tanaka et al., 2004), nerve constriction (Zhang and De Koninck, 2006), ligation and transaction of L5 spinal nerve (Thacker et al., 2009), axotomy, chronic constriction injury (CCI), and spinal nerve ligation (SNL) (Jeon et al., 2009). Moreover, CCL2 is also expressed by Schwann cells at the site of

the injury, as well as by invaded macrophages (Tofaris et al., 2002, Kiguchi et al., 2013). CCL2 preferably binds to CCR2 receptor, while it recognizes also CCR1 and CCR4 (White et al., 2007). CCL2/CCR2 signaling has been implicated in various physiological and pathophysiological processes held in different cell types including neurons, glia, and monocytes (Gao and Ji, 2010). CCL2/CCR2 signaling is considered as main mediator of monocytes/macrophage entry to the nervous system, as CCR2-knockout mice exhibited a significant impairment in macrophage infiltration to the injured sciatic nerve (Siebert et al., 2000). Patrolling circulatory monocytes usually are $CCR2^{low}$/fractalkine receptor $(CX3CR1)^{high}$, however under inflammatory conditions, CCR2 in their surface is upregulated, leading to increased responsiveness to CCL2 (Yang et al., 2014). The monocytes invasion and macrophage accumulation driven by peripheral nerve injury occurs in two distinct compartments – at the site of the injury and in axotomized DRGs. At the site of the injury, activated Schwann cells, mast cells, neutrophils, and macrophages represent the source of CCL2 (Ellis and Bennett, 2013). In DRGs, both neurons and SGC synthesize and release CCL2 in react to peripheral nerve injury (Jung et al., 2008). Interestingly, CCL2 is colocalized with neuropeptides substance P and CGRP, as well as with TRPV1 receptors in DRG neurons, indicating CCL2 synthesis primarily by nociceptive neurons (Dansereau et al., 2008). Monocyte invasion to DRG and to the injury site follows similar timeline – it begins approximately 48 h after the injury, peaks between 7^{th} and 14^{th} day with elevated numbers of monocytes-derived macrophages remaining for at least 28 days after the injury (Lindborg et al., 2018).

Besides its chemoattractant effect on $CCR2^{+}$ circulating monocytes, CCL2/CCR2 signaling is implicated in neuronal functions. CCR2 is expressed by DRG neurons and its expression is upregulated after peripheral nerve injury (Jung et al., 2008). Interestingly, CCR2 is partially colocalized with CCL2 in DRG, suggesting possible autocrine/paracrine manner of CCL2/CCR2 signaling in DRG neurons (Jung et al., 2009). Hence, under pathological conditions, DRG neurons tend to increase their responsiveness to CCL2 and become strongly depolarized by CCL2 application (White et al., 2005). CCL2, synthesized in DRG neurons may also be transported both to the periphery (Bogen et al., 2009), and to the spinal cord through the release from central terminals (Dansereau et al., 2008), thereby contributing to the central sensitization.

As a principal player of innate immune response, recruited macrophages secrete a variety of pro-inflammatory mediators. Along with abovementioned chemokine CCL2, macrophages also release pro-inflammatory cytokines interleukin 6 (IL6), interleukin 1 beta (IL1β) and tumor necrosis factor alpha (TNFα). All these cytokines have well-documented pro-nociceptive effects in the peripheral nervous system, as most DRG

neurons express specific cytokine receptors. For example, IL6 exert its effect on neurons via the formation of complex structure with its soluble receptor sIL6R, which in turn modulates CGRP release by the nociceptor (Opree and Kress, 2000). IL1β acts via its receptor IL1R1, which is mainly expressed by DRG neurons, and may directly sensitize those cells to noxious heat signals through PKC-dependent mechanism (Obreja et al., 2002). TNFα is generally considered a principal cytokine in inflammatory cascade. TNFα is recognized by two receptor types - TNFR1 and TNFR2, which are known to be implicated in nociceptive signaling, as their expression is upregulated in DRG neurons after the nerve injury (Schafers et al., 2003b). TNFα may sensitize nociceptors indirectly, contributing to the development of neuroinflammation by the inducement of the expression of other cytokines (IL1β, IL6, and IL8) (Sommer and Kress, 2004). On the other hand, TNFα is capable of direct nociceptor sensitization, as TNFα application into DRG induces mechanical allodynia (Schafers et al., 2003b). Moreover, following peripheral nerve injury, DRG neurons both with injured afferents and also neighboring neurons, produce TNFα and become sensitized to its excitatory effects as well (Schafers et al., 2003a). To fulfill their pathogen-killing function, invaded macrophages have to produce potentially harmful oxidants, such as reactive oxygen species (ROX) and nitric oxide (NO). ROX production in macrophages is mediated by nicotinamide adenine dinucleotide phosphate oxidase isoform – NOX2. Indeed, peripheral nerve injury-induced neuropathic behavior was significantly attenuated in NOX2-deficient mice, as well as TNFα upregulation in DRG (Kallenborn-Gerhardt et al., 2014). NO produced by nitric oxide synthase (NOS) also modulates nociceptive signaling in DRGs. Usually, NO is produced by neuronal and endothelial isoforms of NOS (nNOS and eNOS). However, under pathological conditions, inducible (iNOS or NOS2) isoform in non-neuronal cells undergoes activation and starts to produce NO in response to the increased cytokine concentration (Wink et al., 2011). Along with cell damage due to oxidative stress, NO also may directly modulate nociceptive signaling by binding to and activating of TRP family channels, namely V1 and ankyrin 1 (TRPA1) channels in nociceptors (Miyamoto et al., 2009).

Neuroimmunological response may be caused not only by mechanical damage of peripheral nerve, but also by neurotoxic changes driven by drug treatment, e.g. cytostatics. Chemotherapy – induced peripheral neuropathy (CIPN), occurring after anti-cancer drug treatment is also characterized by peripheral neuroinflammation. Although peripheral nerves are protected with BNB, the lack of the effective neurovascular barrier in DRGs makes them vulnerable and susceptible to neurotoxicity (Jimenez-Andrade et al., 2008). Systemically administered paclitaxel (PAC) does not penetrate BBB, however it crosses BNB and accumulates in DRGs, where its concentration is the highest when compared to

other neural structures, such as dorsal and ventral roots, sciatic nerve and spinal cord (Cavaletti et al., 2000). Moreover, high PAC concentrations in DRGs sustain up to 10 days after the last injection (Xiao et al., 2011). Growing number of studies demonstrate an important role of peripheral neuroinflammation in the development of CIPN. Peripheral glial cell activation, circulatory monocyte recruitment and macrophage accumulation in DRGs were documented in animal models of CIPN, induced by paclitaxel (Peters et al., 2007), oxaliplatin (Li et al., 2016), vincristine (Kiguchi et al., 2008), and bortezomib administration (Liu et al., 2016).

In addition to abovementioned chemokines and cytokines, damaged tissue and infiltrated leukocytes upregulate the production and release of bradykinin, serotonin, eicosanoids, adenosine triphosphate (ATP), neurotrophins and prostaglandins that contribute to modulation of nociceptive signaling. Taken together, long-lasting peripheral neuroinflammation driven by pro-inflammatory mediators contributes to the generation of ectopic discharges in nociceptors and thus to the development of peripheral sensitization.

2.5.2 Neuroinflammatory changes in the CNS

Persistent ectopic activity of peripheral nociceptors results in prolonged activation of central nociceptive circuits. As it was described before, activity-dependent sensitization of CNS pain-processing neurons and the activation of spinal glial cells are considered the basic mechanisms of central sensitization. Enhanced presynaptic activity in the SCDH is followed by the increased release of glutamate, substance P, ATP, and BDNF from the central terminals, resulting in prolonged depolarization of second-order neurons, release of Mg^{2+} block of NMDA receptors and total enhancement of postsynaptic activity (Ellis and Bennett, 2013). Recently, it became clear that contribution of spinal neuroimmune changes to the development, spread, potentiation, and maintenance of neuropathic pain states was underestimated. Along with the postsynaptic effects of chemokines, cytokines, and nucleotides, released by primary afferents, they act directly on spinal glial cells inducing their activation. It was demonstrated, that glial activation is necessary for enhanced pain transmission in every animal model tested to date. Additionally, while BSCB normally protects spinal cord from circulatory immune cells extravasation, peripheral nerve injury may cause CCL2-mediated transient BSCB breakdown (Beggs et al., 2010, Echeverry et al., 2011), allowing the entry of immune cells

(macrophages and T-cells) as well as other potentially damaging substances from the circulation (immunoglobulin G-IgG).

Microglia is considered the resident macrophages in the CNS. Those glial cells exhibit marked phenotypic shape-shifting properties in reaction to the composition of surrounding microenvironment. Upon peripheral nerve injury, ATP, colony-stimulating factor 1 (CSF1), chemokines, and proteases, released by affected neurons, can directly activate spinal microglia. Simultaneously, microglia upregulates the expression of surface receptors detecting these substances that results in the enhancement of inflammatory cascade (Ji et al., 2016). Activated microglia rapidly proliferates, changes its shape from regular to amoeboid, and exerts new properties, such as migration, antigen presentation, and phagocytosis. Along with morphological and functional changes, the hallmark of microglia activation is the upregulation of the complement receptor 3, also known as cluster determinant (CD) 11b or integrin αM which is recognized by the antibody OX42. Recently, it has been suggested that the main molecular transducer for microglia activation is chemokine CCL2 (Gao and Ji, 2010, Van Steenwinckel et al., 2011, Beggs et al., 2012). Following peripheral nerve injury, CCL2 is produced by affected DRG neurons, transported through central axon terminals, and released in activity-dependent manner during depolarization into the synaptic cleft, where it acts as pro-nociceptive neuromodulator (Van Steenwinckel et al., 2011). Upon binding to CCR2, expressed by resident microglia and, later, bone-marrow derived monocytes (Zhang et al., 2007), CCL2 induces intracellular cascade, leading to the synthesis of several pro-inflammatory markers. CCL2/CCR2 signaling is crucial for microglia activation as spinal injection of CCL2 neutralizing antibody or genetic ablation of CCR2 prevented injury-induced microgliosis (Zhang et al., 2007, Thacker et al., 2009). CCL2 stimulation leads to the surface expression of P2X purinergic receptor 4 (P2X4) in microglia (Toyomitsu et al., 2012). Along with CCL2, CCL21, IFNγ, and tryptase were shown to modulate P2X4 receptors expression in microglia. $P2X4^+$ microglia, activated by injured neurons-derived ATP, is essential for the development of pain hypersensitivity after peripheral nerve injury, since P2X4R-null mice do not develop pain hypersensitivity after peripheral nerve injury (Ulmann et al., 2008, Beggs et al., 2012).

Activated microglia is the main source of pro-inflammatory mediators in the SCDH - TNFα, IL1β, BDNF or cyclooxygenase (COX)-mediated expression of prostaglandin E_2 (Coull et al., 2005). For example, TNFα/TNF(R1 or R2) signaling utilize intracellular cascade resulting in activation of transcription factors activator protein 1 (AP-1) and NFκB-mediated pathways, engaging further the transcription and release of cytokines and enzymes (COX2 and iNOS) (Cao and Zhang, 2008). Besides the expected facilitation of

inflammatory cascade, these substances may directly modulate synaptic transmission in the SCDH. Cytokine TNFα is known to enhance the amplitude of postsynaptic glutamate-induced excitatory currents (Kawasaki et al., 2008) or presynaptically facilitate spontaneous transmitter release (Spicarova et al., 2011). IL1β also modulates excitatory synaptic transmission simultaneously reducing inhibitory transmission (Kawasaki et al., 2008). Microglia-derived BDNF contributes to the establishment of disinhibition in the SCDH via the downregulation of the K^+-Cl^- cotransporter KCC2 and subsequent reducement of GABA hyperpolarizing effect due to the anion gradient shift (Bernal and Roza, 2018).

Microgliosis followed by the increased production and the release of pro-inflammatory mediators was demonstrated not only after peripheral nerve injury, but also in other neuropathic pain models, such as streptozocin-induced diabetic neuropathy, bone cancer pain (Hu et al., 2012) or CIPN (Lees et al., 2017). For example, paclitaxel (PAC) emulate the properties of damage-associated molecular patterns, thus, may directly activate rodent Toll-like receptor 4 (TLR4) receptors (Li et al., 2014, Li et al., 2015). Microglial TLR4 activation by PAC represents the possible mechanism of CCL2 upregulation and subsequent macrophage recruitment to DRGs during CIPN (Zhang et al., 2016). TLR4 is abundantly expressed by spinal microglia; its activation is followed by a nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling downstream resulting in the transcriptional regulation of pro-inflammatory mediators and other potent molecules (Lehnardt et al., 2003). TLR4-knockout or mutated mice exhibit attenuated neuropathic pain behavior and reducement in the expression of TNFα, IL1β, and IFNγ after the peripheral nerve injury (Tanga et al., 2005).

Notably, the application of minocycline, a second-generation tetracycline, which is a potent inhibitor of microglia activation, prevents the development of neuropathic behavior after the nerve injury. However, minocycline treatment fail to reverse already established neuropathic pain, indicating the essential role of activated microglia for the development of neuropathic pain, but not for its maintenance (Ellis and Bennett, 2013).

While the activation of microglia is rapid and transient, the activation of another glial cell type in the CNS - astrocytes - is often delayed and lasts much longer. Astrocyte activation - astrogliosis - occurs several days after the insult (Romero-Sandoval et al., 2008). Upon activation, astrocytes also undergo morphological changes (proliferation and differentiation), enlarge, upregulate the expression of intermediate filament glial fibrillary acidic protein (GFAP) and the calcium-binding peptide S100β, and engage the release of pro-inflammatory substances (Tanga et al., 2004). Astrocytes may be activated by the same range of stimuli as microglia does and/or by the products of earlier activated

microglia; they adopt the function of antigen presentation, promoting thus T helper 2 (Th2) responses (Murray et al., 2014). Activated astrocytes produce and release CCL2, CXCL1, ATP, glutamate and IFNα, which are known both to mediate nociceptive signaling in the SCDH and to facilitate the neuroimmune response via bilateral positive feedback signaling with microglia (Ji et al., 2016). Considering the crucial role of astrocytic functions, such as neurotransmitter recycling, formation of BSCB, regulation of extracellular ion balance, and synaptic transmission modulation, the functional shift to the activated state leads to the impairment in the executive abilities of astrocytes. Namely, activated astrocytes fail to maintain extracellular K^+ and glutamate concentration balance, resulting in neuron hyperexcitability (Ji et al., 2013). Astrocytes also directly communicate with neurons through gap-junctions that enable paracrine modulation of neuronal excitability by activated astrocytes (Chen et al., 2014a).

Regarding chronological events in cellular activation, the sequential activation of MAP kinase ERK in neurons, then microglia and later astroglia was demonstrated in SNL model (Zhuang et al., 2005). On the other hand, in paclitaxel-induced CIPN model, astrocyte activation was observed in the absence of microgliosis and was detected already 4 hours after PAC treatment and lasted for 28 days (Zhang et al., 2012). Nonetheless, astrocytes are considered the main mediator involved in the maintenance of pain hypersensitivity, as GFAP deficient mice do develop pain-like behavior after peripheral nerve injury, but it lasts for shorter time, that in wild-type mice. Moreover, GFAP-antisense treatment reverses established pain hypersensitivity 6 weeks after peripheral nerve injury (Kim et al., 2009).

Finally, the activation of microglia and/or astrocytes was reported in higher brain centers (hypothalamus and PAG) and brainstem rostral ventromedial medulla (RVM) after peripheral nerve injury (Wei et al., 2008, Takeda et al., 2009), in anterior cingulate cortex after the paclitaxel treatment (Masocha, 2015) and in thalamus, somatosensory area S1, and PAG after the oxaliplatin treatment (Di Cesare Mannelli et al., 2014). These findings indicate that neuroimmune changes modulate central pain processing not only on the SCDH level, but also supraspinally, contributing thus to neuropathic pain establishment.

2.5.3 Macrophage polarization

It is now well documented that damage or injury to the nervous tissue is followed by the recruitment of circulating monocytes to the inflamed tissue. Once inside the tissue,

monocytes rapidly give rise to macrophages, which are remarkably plastic in their response to surrounding microenvironment. The variety of receptors expressed by macrophages, enable them to sense even minor changes in homeostasis. In reaction to these cues, macrophages exert transcriptional and translational response, creating a whole spectrum of activation phenotypes (Martinez et al., 2013). The debate about proper description of macrophage activation states is open currently and some final consensus is still missing (Murray et al., 2014). Nevertheless, the most prominent and often used is the M1/M2 nomenclature of macrophage activation. Originally proposed by Mills and colleagues in 2000 (Mills et al., 2000) and further developed and modified by others, it is based on the differences between the macrophage response to T helper 1 (Th1) cytokine IFNγ, creating M1 phenotype, and to Th2 cytokines IL4 and IL13, giving rise to M2 phenotype. Macrophage M1/M2 phenotypes are viewed as two extremes of a spectrum of activation states, following the nomenclature of T helper cells (Weigert et al., 2018). Interestingly, M1/M2 classification is also used to describe the activation states of microglia in the CNS, reflecting macrophage and microglia functional similarities (Lampron et al., 2013, Franco and Fernandez-Suarez, 2015). However, M1/M2 classification is still highly debated. It is not completely clear, if macrophages are capable of innate M1 to M2 transition or how do they respond to a mixture of environmental cues *in vivo* (Murray et al., 2014). Indeed, while macrophage polarization is relatively well described *in vitro*, it is clear that *in vivo* recruited monocytes are exposed to a mixture of environmental signals, which vary in different tissues and conditions, creating complex phenotypes and mixed populations of macrophages. Although this classification is currently considered as insufficient to describe whole spectrum of activation states, we decided to use M1/M2 paradigm in this work in order to simplify the terminology. In terms of nociception, we consider M1 type as pro-nociceptive, and M2 type as those, which exert anti-nociceptive effects (pro-resolving, anti-inflammatory, and antagonistic to M1 effects).

Briefly, M1 macrophages, also called “classically activated”, are those stimulated with IFNγ, TNFα or TLR ligands, such as lipopolysaccharide (LPS), exerting strong pro-inflammatory profile. When macrophages polarize to M1-like state, their transcriptional signature is characterized by the activation of signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 5 (IRF5) (Weigert et al., 2018). The downstream signaling results in abundant expression and release of pro-inflammatory cytokines and chemokines, as well as ROS and nitrogen species, which contribute to the development and maintenance of inflammation (described in detail in previous chapter). In contrast, IL4 or IL13 stimulated macrophages, so-called M2 or

"alternatively activated", generate an alternative set of signaling molecules, which functionally oppose the repertoire of M1 macrophages. The dominant signaling pathways in M2 macrophages are via phosphorylation of STAT6 and IRF4 activation (Loane et al., 2009, Murray et al., 2014). M2 phenotype is also characterized by the expression of specific phagocytic mannose receptor C type 1 (CD206), the scavenger receptor CD163, and anti-inflammatory mediators (Loane et al., 2009). IL4- and IL13-stimulated macrophages induce the expression of M2 chemokines (CCL17, CCL18, CCL22 and CCL24) and cytokines (IL1ra, TGFβ and IL10) and upregulate MHC class II molecules (Gordon, 2003, Franco and Fernandez-Suarez, 2015). Notably, the expression of Arginase 1 (ARG1), until recently considered as a marker of M2 macrophages, is now taken with precaution, as ARG1 expression was documented in LPS-stimulated macrophages, although in relatively lower amounts (Murray et al., 2014). However, in contrast to M1 macrophages, M2 subtype expresses ARG1 constitutively, thus preferring hydrolysis of L-arginine to L-ornithine and urea (Mantovani et al., 2002, Ruytinx et al., 2018). In M1-type macrophages, L-arginine is primarily consumed by iNOS to produce NO, hence, ARG1 upregulation in M2 phenotype results in substrate competition and prevention of NO synthesis (Munder et al., 1999).

M2 polarization type of macrophages may be induced not only by IL4 or IL13 signaling, but also by IL10 (Gordon, 2003). In contrast to IL4/IL13 signaling, IL10 binds to different receptor IL10R, which interacts with the Janus kinase (JAK) family tyrosine kinases, activating further STAT3 in macrophages (Moore et al., 2001). Therefore, IL10-activated macrophages are often referred as different subtype of alternatively activated macrophages - M2c type (Franco and Fernandez-Suarez, 2015). For example, IL10/IL10R signaling leads to more potent inhibition of LPS-induced TNFα production and iNOS activation, when compared to IL4 and IL13 effect (Gordon, 2003). Moreover, IL10 utilizes transcriptional and posttranscriptional mechanisms to downregulate the production of wide range of pro-inflammatory molecules, as well as MHC class II antigens, thereby contributing to the attenuation of immune response and inflammation (Moore et al., 2001).

Several lines of evidence indicate that inhibition of M1-dominant molecules and upregulation of M2-specific signaling attenuate neuropathic pain (Kiguchi et al., 2017, Montague and Malcangio, 2017). From pharmaceutical point of view, targeted manipulation of gene expression in macrophages and their profile shift from pro-inflammatory to anti-inflammatory state represents a valuable therapeutic benefit. First, the unpleasant inflammatory phase may be shortened with this approach. Secondly, it may prevent the robust neural damage formation and chronic malfunction establishment.

Moreover, enhanced anti-inflammatory cytokine (e.g. IL10) production may lead to tissue repair, which also represents a therapeutic potential.

2.5.4 The role of PPARγ in macrophage polarization

Peroxisome proliferator-activated receptors (PPARs) belong to a family of nuclear receptors, which are known for their role in metabolism as lipid activated transcription factors. PPAR family consists of three types of receptors - α, β/δ, and γ, which are distributed differently and exert different functions. Upon activation by endogenous or synthetic ligands, PPARs form heterodimers with retinoid X receptors (RXRs) and bind to specific response elements (PPREs) in the promoter region of target genes (Freitag and Miller, 2014). Recently, PPARγ were intensively studied for their role in the macrophage polarization. A growing number of studies confirm that PPARγ activation triggers macrophage polarity shift from M1 (pro-inflammatory) to M2 (anti-inflammatory) phenotype, thus reducing local neuroinflammation and neuropathic pain behavior (Churi et al., 2008, Hasegawa-Moriyama et al., 2012). Moreover, PPARγ is upregulated in activated macrophages and their migration, proliferation and phagocytic activities may be inhibited by PPARγ agonists (Freitag and Miller, 2014). Jiang and colleagues were first to demonstrate that PPARγ agonists block the production of TNFα, IL6, and IL1β in cultured monocytes (Jiang et al., 1998). Treatment with PPARγ agonists was also effective in inhibition of CCL2 production by LPS-stimulated cultured microglia and, to a lesser extent, by astrocytes (Storer et al., 2005). Along with the regulation of CCL2 production, CCR2 expression is also under PPARγ control as promoters for CCR2 expression may be repressed by ligand-bound PPARγ (Chen et al., 2005). The molecular mechanism of PPARγ-mediated inhibition of gene expression is called transinhibition, when PPARγ targets the transcription factors (NFκB, AP1, and STAT) without direct interaction with DNA (Freitag and Miller, 2014). PPARγ signaling is also required for IL4-mediated changes in gene expression, while PPARγ expression is upregulated in IL4-dependent manner (Nelson et al., 2018). Another anti-inflammatory effect of PPARγ is mediated by their ability to directly bind the PKCα and consequently cease PKC-mediated iNOS activation in macrophages (von Knethen et al., 2007). Moreover, ligand-bounded PPARγ were shown to enhance the expression of anti-inflammatory molecules IL10 and ARG1 in murine LPS-stimulated macrophages (Bouhlel et al., 2007, Su et al., 2017). Interestingly, PPARγ agonists also affect other cell types, such as astrocytes, microglia, oligodendrocytes and neurons (Bernardo et al., 2000, Bernardo and Minghetti, 2008, Song et al., 2016).

A broad range of PPARγ ligands includes natural and synthetic agonists. Natural, or endogenic, agonists are long chain fatty acids and the cyclopentenone prostaglandin 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2); synthetic compounds include thiazolidinediones (TZDs) as rosiglitazone and pioglitazone; and some nonsteroidal anti-inflammatory drugs (NSAIDs). Their therapeutic efficacy was confirmed in numerous pathological conditions, such as type II diabetes (Chigurupati et al., 2015) and cardiovascular diseases (Cheang et al., 2015). Currently, the anti-inflammatory effects of PPARγ agonists are extensively studied in animal models of neurological diseases - Parkinson's disease (Breidert et al., 2002), Alzheimer's disease (Jiang et al., 2008), amyotrophic lateral sclerosis (Schutz et al., 2005) and also in spinal cord injury model (Park et al., 2007). Additionally, PPARγ agonists were tested for their ability to modulate neuropathic pain development after peripheral nerve injury (Churi et al., 2008, Costa et al., 2008, Maeda et al., 2008, Freitag and Miller, 2014). Palmitoylethanolamide (PEA), an endogenous ligand of PPARα, is now used in some European countries under the names Normast® or PeaPure® as a dietary supplement prescribed for the treatment of neuropathic pain and inflammation (Freitag and Miller, 2014). Notable, Costa and colleagues claimed that PEA utilized cannabinoid receptor type 1, TRPV1 and PPARγ to mediate its antinociceptive effects (Costa et al., 2008). Although therapeutic efficacy of PPARγ agonists was confirmed, many of them had been withdrawn from the drug market due to their severe side effects, namely, increased risk of myocardial infarction (Lincoff et al., 2007, Nissen and Wolski, 2007). Nowadays, effective second-generation PPARγ agonists that exert similar therapeutic benefits without unwanted side effects represent a perspective target in drug development.

2.6 The role of renin-angiotensin system and sartans in neuroinflammation

The renin-angiotensin system (RAS) is a principal hemodynamic regulator. This complex system regulates blood pressure, vasoconstriction, and sodium intake and potassium excretion in mammals. Renin, released by renal juxtaglomerular cells, cleaves glycoprotein angiotensinogen produced in liver to angiotensin I. Angiotensin I is subsequently converted to an active substance angiotensin II (Ang II) by the angiotensin converting enzymes (ACE and ACE2) (Benigni et al., 2010). ACE, predominantly expressed in endothelial surface of lung vessels, mediates two principal processes: conversion of Angiotensin I to Ang II and hydrolysis of bradykinin to its inactive form (Fleming, 2006).

Ang II, the essential effector molecule of the RAS, induces the release of catecholamines from nerve terminals and adrenal gland, increases thirst, salt retention and vasoconstriction, thus contributing to the development of hypertension (Crowley et al., 2006). Ang II exerts its functions via binding to two types of G protein-coupled receptors: angiotensin type 1 and type 2 receptors (AT1R and AT2R). AT1R receptors mediate the majority of physiological effects of Ang II, while AT2R receptor is highly expressed in developing fetal tissues and decreases after birth. AT1R are abundantly expressed in different tissues as kidney, heart, vascular smooth muscle, and pituitary gland (Benigni et al., 2010). The expression of AT1R and other components of RAS, was also confirmed in brain structures, where Ang II may be synthesized by brain cells independently of peripheral sources, indicating the presence of local RAS (Paul et al., 2006). Besides the involvement in central blood pressure and homeostasis regulation, brain local RAS was also described as a potent central regulator of sensory information processing. For example, Ang II was shown to modulate baroreceptor and chemoreceptor pathways via interaction with AT1R in the brainstem nucleus of the solitary tract (Paton and Kasparov, 2000). Moreover, RAS in PAG is also involved in modulation of nociceptive signaling (Pelegrini-da-Silva et al., 2005). These findings inspired Pavel and colleagues to examine in detail the role of Ang II in the sensory system. Using quantitative autoradiography and *in situ* hybridization for AT1R and AT2R detection, they described a substantial expression of AT1R in superficial SCDH, neuronal clusters in DRGs and throughout the sciatic nerve (Pavel et al., 2008). Notably, the expression level of AT2R in these structures was inconsistent or below the detection threshold. High density of AT1R was also measured in large spinal cord arteries, suggesting the involvement of AT1R in the regulation of blood flow in the CNS.

Ang II and AT1R signaling are intensively studied for their role in the regulation of inflammation (Suzuki et al., 2003). Several studies indicated that treatment with ATR blockers (ARBs) has a neuroprotective effect in neurodegenerative diseases, such as the Alzheimer's (Wang et al., 2007) and the Parkinson's (Mertens et al., 2010). AT1R blockers were also tested with encouraging results in such pathophysiological conditions as atherosclerosis (Yamamoto et al., 2015), diabetes (Nakamura et al., 2009) and other inflammatory diseases. In the CNS, astrocytes are considered as primary target of Ang II, as these glial cells express AT1R and AT2R, both isoforms of ACE and produce angiotensinogen (Gallagher et al., 2006). In astrocytes, Ang II/ATR signaling leads to the production of IL6 via JAK2-dependent phosphorylation of STAT3 mechanism (Kandalam and Clark, 2010). Additionally, Ang II elicits cellular responses also via the activation of nuclear transcription factors AP-1 and NFκB, which further trigger the production of pro-

inflammatory proteins as adhesion molecules, cytokines, and chemokines (Suzuki et al., 2003). On the other side, Ang II may directly activate immune cells, thus modulating their principal functions - chemotaxis, proliferation, and differentiation. Moreover, Ang II may induce alterations in vascular permeability, as AT1R signaling in endothelial cells is known to induce the production of prostaglandins (leukotriene C4, PGE2 and PGI2) and vascular endothelial cell growth factor (Suzuki et al., 2003).

Among all ARBs, sartans are the most frequently prescribed. The sartans have a low incidence of adverse effects, such as headache, upper respiratory infection, back pain, muscle cramps, fatigue, and dizziness (Israili, 2000). Sartan family consists of losartan, telmisartan, valsartan, candesartan, irbesartan, and eprosartan. These drugs have different molecular structures, which influence their pharmacokinetic properties, such as bioavailability, biotransformation, receptor affinity, and efficacy. Losartan was the first clinically approved among sartans. Unlike other sartans, it is a prodrug, which upon systemic administration is metabolized in liver by cytochrome P450 3A4- and 2C9-subunits to two metabolites: EXP3174 and EXP3179 (Oparil, 2000, Song and White, 2000). EXP3174 has been described as proper AT1R blocker, acting as a competitive antagonist (Lo et al., 1995, Stearns et al., 1995), while EXP3179 does not interfere with any Ang II binding sites (Kramer et al., 2002). However, EXP3179 exhibits molecular homology to a COX2 inhibitor indometacin, thus inhibits COX2-mediated thromboxane A2 and prostaglandine F2α synthesis (Kramer et al., 2002). Moreover, Schupp and colleagues demonstrated that EXP3179, but not EXP3174, markedly stimulates PPARγ activity *in vitro* in a manner of partial agonist (Schupp et al., 2006). Further, it was shown that chronic oral treatment with losartan leads to serum levels of EXP3179 that are sufficient to activate PPARγ (Kappert et al., 2009). Later, PPARγ agonistic properties were also confirmed for other sartans - telmisartan and irbesartan (Haraguchi et al., 2009, Iwai et al., 2011). However, comparative study, focused on the effects of different sartans on chemotactic migration of human monocytes, revealed that treatment with EXP3179 had significant effect on monocyte migration that was similar to pioglitazone, the PPARγ full agonist. Moreover, unlike any other sartan or pioglitazone, EXP3179 was able to reduce monocyte migration even in low dosages, which were similar to those in losartan treated patients (Kappert et al., 2009).

Currently, treatment strategies involving RAS are of particular interest. Two research groups reported analgesic effects of small molecule AT2R antagonists in a peripheral nerve injury and antiretroviral neuropathic pain models in rats (Smith et al., 2013, Smith et al., 2014), as well in postherpetic neuralgia patients (Rice et al., 2014). In particular, the advanced study of Rice and coll. in a randomized, double-blind, placebo-

controlled Phase II clinical trial, showed a superior relief of postherpetic neuralgia with no serious side effects in patients treated with EMA401, a small molecule AT2R antagonist, when compared with patients who received placebo (Rice et al., 2014).

3. AIMS OF THE THESIS

The general goal of this thesis was to investigate the mechanisms underlying the development of pathological pain states using acute and chronic pain models with *in vitro* and *in vivo* approaches. The main part of this thesis is focused on the anti-inflammatory role of systemic losartan treatment in neuropathic pain models. The other part is devoted to the role of TRPV1 receptor signaling in the development of central sensitization.

The specific aims are the following:

1) to study the effect of systemic losartan treatment on the development of peripheral neuropathy after peripheral nerve injury;

2) to describe the effect of systemic losartan treatment on PIPN-induced neuropathic changes and to investigate the possible underlying mechanism of losartan's action;

3) to evaluate the effect of paclitaxel treatment on spinal neuronal activation and to describe the role of TRPV1 receptors in this process;

4) to characterize the involvement of TRPV1 receptor signaling in CCL2-induced spinal neuronal activation and in the development of acute pain hypersensitivity in rats with intrathecal CCL2 application.

4. MATERIALS AND METHODS

4.1 Experimental animals

All experiments were approved by the Animal Care and Use Committee of the Institute of Physiology CAS and were carried out in accordance with the guidelines of the International Association for the Study of Pain, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the European Communities Council Directive of 24 November 1986 (86/609/EEC) and EU Directive 2010/63/EU for animal experiments. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

All experiments were conducted in male Wistar rats (Institute of Physiology CAS breeding program with government permission for breeding laboratory animals). For *in vitro* experiments, acute spinal cord slices were prepared from juvenile male Wistar rats (P21-P23). All *in vivo* experiments were conducted in adult male Wistar rats, weighing between 250 and 300g at the beginning of the experimental protocol. Animals were kept on a 12-h light/dark cycle in plastic cages with soft bedding, in an environment with adequate temperature and ventilation (22 ± 1^0C) with pellet food and water *ad libitum*. The experiments were carried out during the light phase of the cycle.

4.2 Assessment of mechanical and heat sensitivity

In all the experimental groups, baseline behavioral data were obtained before the induction of peripheral neuropathy model or any other experimental procedure. Mechanical paw withdrawal threshold (PWT) was assessed on hind paws using electronic dynamic plantar von Frey aesthesiometer (IITC Inc Life Science, Model 2390 Series). The mechanical withdrawal threshold was the pressure exerted (in grams) that triggered the paw withdrawal. Each stimulus was applied 4 times with 5 min between trials. Results from each hind paw were averaged and SEMs calculated. Paw withdrawal latencies (PWL) to radiant heat stimuli were determined for both hindpaws. The rats were placed under non-binding, clear plastic cages on a 3-mm thick glass plate and left to adapt for at least 20 min. The radiant heat was applied to the plantar surface of each hindpaw until a deliberate escape movement of the paw was observed. For study with SNL-operated animals, a focused light source with a halogen bulb was used to deliver heat stimuli (50 W, Dittel).

The PWL was measured by a digital watch with a manual release switch electrically connected with the heat source. CCL2-induced acute thermal hyperalgesia was measured using a plantar test apparatus (Ugo Basile, Italy). A 30 s cutoff time was imposed on the stimulus duration to prevent any tissue damage. The PWL were tested 4 times for each hindpaw with 5-min intervals between the trials. Results from each hind paw were averaged and SEMs calculated.

4.3 Surgical procedures

4.3.1 Spinal nerve ligation (SNL)

The spinal nerve ligation (SNL) was performed on the left side of the rat under ketamine (100 mg/kg i.p., Narketan, Zentiva) and xylazine (25 mg/kg i.p., Xylapan, Zentiva) anesthesia. The day of the surgery is referred as day 0. The ligation of L5 spinal nerve was performed similar to the method described by Kim and Chung (Kim and Chung, 1992). The fur was shaved and the skin was disinfected with antiseptic (Jodisol, Spofa), an incision was made on the left side of the spine at the L4-S1 level. The left transverse process of L6 vertebra was first removed; L5 spinal nerve was exposed and then tightly ligated with 5.0 silk thread. Complete hemostasis was confirmed and the wound was surgically closed in layers.

4.3.2 Intrathecal catheter implantation

Catheters were made with two polyethylene tubes of different size PE-5 and PE-10. PE-10 tube was first bended to the necessary form and then connected on one side with the PE-5 tubing with epoxy glue. Prepared catheter was filled with sterile saline. Catheter implantation was performed under deep anesthesia simultaneously with the SNL operation. The fur was shaved and the skin was disinfected with antiseptic (Jodisol, Spofa), then longitudinal incision was made at the region between L3-S1 spinal processes. Spinal muscles were retracted and small opening was made in dura mater with microforceps at the intravertebral area L5-L6. The PE-5 end of the catheter was inserted to the subarachnoidal space and fixed to the vertebral column with dental cement. The wound was then surgically closed with sutures. PE-10 end of the catheter was externalized in the

occipital region and sealed. Each animal was allowed to recover from the surgery for 24 hours before any behavioral testing.

4.3.3 Drug administration

For the intrathecal application of CCL2 or SB366791, 10 µl of saline, 10 µl of CCL2 solution (1.4 µg) or 15 µl of SB366791 solution (0.43 µg) followed by 45 µl of saline, were delivered via lumbosacral intrathecal catheter. In another set of experiments, intrathecal injections were performed by a direct lumbar puncture between the L4 and L5 vertebrae. Briefly, rats were injected with saline, CCL2 (1 µg), and/or SB366791 (0.5 µg) in a volume of 30 µl under the isoflurane anesthesia (Forane, Abb Vie s.r.o., Czech Republic).

Losartan was administered *per os* (*p.o.*) or intrathecally (*i.t.*). For the *p.o.* administration, losartan (Lozap, Zentiva) was dissolved in the drinking water for the use during the experiment. The losartan solution was changed daily, the amount consumed was registered and losartan concentration adjusted to reach the average dose of 100 mg/kg/day. For the *i.t.* application - 10 µl of 20 µM solution of losartan (Losartan Potassium, Tocris) was injected via lumbosacral catheter under isoflurane anesthesia (3%, Forane, AbbVie). This was followed by 40 µl of saline administered into the catheter to clear its dead space. *I.t.* injections were performed daily at the same time starting from the day 0 after the surgery or after the behavioral testing.

Paclitaxel was administered via intraperitoneal (*i. p.*) injections (5 x 2 mg/kg) on five alternate days. Rats in the control group received *i. p.* injections of the vehicle (Kolliphor EL (Sigma Aldrich, Germany)/ethanol, 1:1) on the same 5 alternate days.

4.4 Immunohistochemistry on DRGs and dorsal roots and data quantification

The rats were deeply anesthetized with ketamine (100 mg/kg, Narketan, Zentiva)/xylazine (25 mg/kg, Xylapan, Zentiva) combination, perfused intracardially with a saline followed by ice cold 4% paraformaldehyde. L5 DRGs and adjacent dorsal roots (DR) were removed and post-fixed at 4°C for 24 hours, cryoprotected with sucrose overnight, and cut in cryostat at 16 µm. These sections were then processed for CD68

immunohistochemistry. Briefly, sections were blocked with 3% normal donkey serum for 30 min at RT and incubated overnight at 4°C with mouse anti-CD68 (1:200; Serotec, Raleigh, NC) primary antibody in 1% NDS with 0.3% Triton X-100. The slides were then washed for 30 min in NDS and exposed to a donkey anti-mouse secondary antibody Alexa Fluor ® 488 (1:400, Jackson Immuno Research Inc, USA) for 2 hours. All sections were visualized and captured using fluorescence microscope equipped with a digital camera system (Olympus BX53). For every section, the region of interest (ROI) was outlined and measured (in pixels). For DRG sections, only regions of the sensory ganglia containing sensory neuronal cell bodies (excluding nerve fibers) were outlined. Area of CD68-immunoreactive (IR) cell bodies in this region was measured using ImageJ software (NIH, USA). IR/ROI ratios were calculated and expressed as percentage (IR%).

4.5 Real Time qPCR

The animals were deeply anesthetized with 3% isoflurane (Forane®, Abb Vie s.r.o., Czech Republic) and tissues of interest were briefly removed, frozen in liquid nitrogen and stored at – 80°C. The total RNA from the spinal cord dorsal horn and DRGs was isolated with the commercially available kit RNeasy Mini (Qiagen, Germany) according to the manufacturer's protocol and quantified by spectrophotometry using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was removed during RNA purification with on-column digestion of DNA (RNase-free DNase set, Quiagen, Germany). Reverse transcription to cDNA was performed using ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA). Quantitative RT-PCR was carried out using a Viia 7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), 5x Hot Firepol Probe QPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) and TaqMan Assays (Life Technologies) specific for the studied transcript. The following assays were used: Arg1 (for Arginase 1, Cat. No. Rn00691090_m1), Actb (β-actin, Rn00667869_m1), Ccl2 (CCL2, Rn00580555_m1), Ccr2 (CCR2, Rn01637698_s1), Fcgr1a (CD64, Rn01762682_m1), Cd68 (CD68, Rn01495634_g1), Gfap (GFAP, Rn00566603_m1), Il10 (IL10, Rn01483988_g1), Il6 (IL6, Rn01410330_m1), Nos2 (iNOS, Rn99999069_mH), Cybb (NOX2, Rn00576710_m1), Itgam (CD11b, Rn00709342_m1), Tnf (TNFα, Rn00562055_m1), and Pparg (PPARγ, Rn00440945_m1). β-actin was chosen among four genes (β-actin, GAPDH, HPRT, 18S) as a proper housekeeping gene for our experiments (NormFinder software, MOMA, Denmark). Fold

differences of mRNA levels over vehicle control were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.6 Protein assay

The samples of the DRGs and SCDH for the protein assay were collected at the day 21 after the first PAC injection. The Arginase 1 protein levels were measured by the ELISA kit (Rat Arg1 ELISA Kit, MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. Rats were deeply anesthetized with isoflurane; tissues were removed and immediately frozen in liquid nitrogen, then stored at – 80°C. Prior to ELISA measurement, tissue samples were homogenized in PBS and processed for protein quantification using Pierce ™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

4.7 Western blot assay

Animals were deeply anesthetized with *i.p.* injection of ketamine (100 mg/kg, Narketan, Zentiva) and xylazine (25 mg/kg, Xylapan, Zentiva) for the SNL-model study and with 4 % isoflurane (Forane®, Abb Vie s.r.o., Czech Republic) for the paclitaxel-induced peripheral neuropathy (PIPN)-model study. Briefly, L3-L5 DRGs and L3-L5 spinal cord section were removed. In SNL-operated animals, L4-L5 spinal segments were divided into left (ipsilateral) and right (contralateral) parts with sterilized blade, and further used for immunoblotting. For the PIPN study, dorsal parts of L3-L5 spinal segments were used. The samples were frozen and stored at -80°C. Each sample was mechanically homogenized, centrifuged, and supernatant protein concentration was determined by Bradford method (BioRad protein assay). Protein samples were boiled for 3 min and separated by SDS-PAGE 4-10% Bis acrylamide (Serva)-Tricine (Sigma-Aldrich) gel. Proteins were then electrotransferred onto a nitrocellulose membrane (0.2 μm, BioRad). The membrane was first saturated by incubation in blocking solution (5% bovine serum albumin and 0.1% Tween 20 in TBS) for 20 min and was then incubated overnight at 4°C with monoclonal mouse or polyclonal rabbit anti-β-actin (1:1000 or 1:500 respectively, loading control) and monoclonal mouse anti-CD68 (1:1000) or rabbit polyclonal antibodies: anti-TNFα, anti-TNFR1, anti-OX42, anti-GFAP, anti-AT1R, anti-CCL2, anti-CCR2, anti-IL6, anti-GP130 or anti-TRPV1 (1:000, 1:250, 1:500, 1:1000, 1:500, 1:250, 1:250,

1:1000, 1:1000, 1:1000; respectively) in diluting solution. Blots were rinsed 3 times with 0.1% Tween 20 in TBS and incubated for 90 min with fluorocore-coupled goat anti mouse IRdye 800 and goat anti rabbit licor IRdye 680 (1:5000). Blots were rinsed 3 times with 0.1% Tween 20 in TBS, and then were scanned to reveal the protein bands with the Odyssey System Imager (Li-Cor) coupled to acquisition software. Antibodies were provided by Exbio (anti-CCL2, anti-TNFR1, anti-β-actin), BioVision (anti-CCR2), Neuromics (anti-OX42), Abcam (anti-ATR1, anti-TNFα and anti-IL6), Sigma (anti-GFAP) and Li-cor (anti-mouse, anti-rabbit). The immunoreactivity of proteins of interest was compared with β-actin immunoreactivity values controls and quantified based on scanned images of the blots, with Aida image analyzer software (Aida™). Preliminary analyses were performed to check the variability of β-actin (immunoblotting loading control) levels among the animals and the experiments, using One-way ANOVA statistic test. The results did not show any significant change between the experimental group levels in all animals. For SNL study, all values were obtained after first standardization of the raw values to β-actin corresponding values and then normalization using the contralateral spinal cord sample value as reference of 100%. For PINP study, values were obtained after first standardization of the raw values to β-actin corresponding values and then after normalization using the VEH group spinal cord sample value as reference of 100%. All values are expressed as means ± SEM.

4.8 Spinal cord slice preparation

Acute spinal cord slices from juvenile rats (P21 - P23) were prepared according to previously published method (Spicarova and Palecek, 2009). After deep anaesthesia with 4 % isoflurane (Forane®, Abb Vie s.r.o., Czech Republic), the lumbar spinal cord was briefly removed and immersed in 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, 50 sucrose. The spinal cord was then fixed to vibratome stage (Leica VT1200S, Germany) using cyanoacrylate glue in a groove between two agar blocks. Acute transverse slices 200 μm (for *in vitro* study in the Publication D) or 350 μm thick (for Publication C) were cut from the lumbar segments L4-L6, incubated in the dissection solution for 30 min at 37 °C and then stored in incubating solution at room temperature and allowed to recover for 1 h before the experiment. The incubating solution contained (in mM): 127 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 25 D-glucose. Alternate series of free-floating spinal cord sections were assigned to one of 16 blocks; i.e., each of the L4–L6 segments was subdivided into four blocks along the rostro-caudal axis.

4.8.1 Induction of c-Fos expression via incubation with PAC

In PAC study, acute spinal cord slices were 350 μm thick. After 1 h for recovery in the incubating solution at room temperature, some slices were incubated with PAC (100nM) for 60 min (PAC group). Other slices were incubated with TRPV1 antagonists (10 μM) for 10 min, and then PAC (100nM, 60 min) was added to the incubation chamber (SB+PAC and AMG+PAC groups). Another group of slices was incubated with TRPV1 antagonist only (10 μM, 70 min, SB and AMG groups). A control group of slices was incubated with vehicle (DMSO, 2‰, 70 min, CTRL group). This arrangement would allow negative and positive control for each animal. During the incubation time slices were constantly perfused with solutions saturated with 95% O_2 and 5% CO_2 .

4.8.2 Immunohistochemistry for c-Fos and data quantification

Immediately after the incubation, the slices were fixed in 4% paraformaldehyde overnight, replaced with sucrose overnight, and cut in cryostat at 16 μm. Because the surfaces of the slices were likely to be damaged during the preparation and incubation, we trimmed the slices in a cryostat and collected 15-20 serial sections (16 μm thick) only from the middle of the slices. These sections were then processed for immunohistochemistry using SABC method. Briefly, spinal sections were blocked with 3% normal donkey serum for 30 min at room temperature (RT) and incubated overnight at 4°C with anti-c-Fos (anti-rabbit, 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) antibody in 1% NDS with 0.3% Triton X-100. For SABC staining, the sections were incubated for 2 h with biotinylated secondary antibody (1:400) and 2 h with peroxidase-conjugated streptavidin (1:400; Jackson ImmunoReasearch laboratories, Inc., PA, USA) at RT. Finally, the reaction product was visualized with 1.85 mM DAB/0.003% hydrogen peroxide in PBS for 2–5 min (Sigma Aldrich, Czech Republic). Images of the spinal cord were captured, the superficial laminae I/II of the spinal dorsal horn were outlined and measured (in pixels). Area and the numbers of immunoreactive (IR) neuronal nuclei for c-Fos in this region were counted using ImageJ software. For every section in each slice the nubmers of labeled neurons per measured area ratio was calculated (cells/area*1000). Cells/area ratios were averaged for each slice and eight to sixteen slices from different rats were included in each experimental group. The data are represented as mean ± SEM.

The difference between the groups was compared using One Way ANOVA followed by Holm-Sidak post hoc test (SigmaStat® software), the criterion for statistical significance was $p < 0.05$.

4.8.3 Induction of pERK expression via incubation with CCL2

Spinal cord slices for CCL2 study were cut 200 μm thick. After 1 h of recovery, slices were assigned to three experimental groups. One group of slices was incubated with CCL2 (10 nM for 10 min); another group was incubated with TRPV1 antagonist SB 366791 (10 μm for 5 min), then CCL2 (10 nM for 10 min) was added to incubation chamber. A control group of slices did not receive any further treatment. During the incubation time slices were constantly perfused with solutions saturated with 95% O_2 and 5% CO_2.

4.8.4 Immunohistochemistry for pERK1/2 and data quantification

Lumbar spinal cord slices (200 mm thick) were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h immediately after the incubation. Immunostaining for pERK1/2 was performed by the streptavidine-biotine peroxidase complex method. First, the slices were washed with a solution of 0.1 M phosphate-buffered-saline (PBS) three times for 10 min and then incubated in a blocking solution of 3% normal donkey serum (NDS) for 30 min at room temperature. The slices were then incubated with primary antibody solution (Rabbit, 1:500, Cell Signaling Technology) at 4 °C overnight. After the incubation, the tissue sections were washed in 1% NDS three times for 10 min and incubated for 2 h at room temperature in biotinylated secondary antibody solution (a 1:400 dilution of biotin-SP-conjugated donkey anti-mouse IgG; Jackson ImmunoResearch Laboratories, Inc., USA). Next, the sections were washed in PB twice for 10 min and incubated in peroxidase-conjugated streptavidin (1:600, Jackson ImmunoResearch) for 2 h at room temperature. The reaction product was visualized with 0.01% hydrogen peroxide and 0.05% diaminobenzidine as the chromogen. After rinsing in 0.1MPB for 10 min, the sections were mounted onto gelatinized slides and coverslipped. The spinal cord sections were photographed with an Olympus BX-80 and 5Mpix Digital camera in one image. Two symmetrical areas on each side in the superficial dorsal horn (laminae I-II) were selected for analysis by an experimenter blinded to the treatment group. The

numbers of pERK positive cells from the left and right superficial DH in three spinal cord sections were averaged, and then the results from the individual animals were averaged. All data were expressed as mean ± SEM and one-way ANOVA followed by a post hoc test (Student-Newman-Keuls Method) was used for statistical comparisons.

4.9 Data analysis

All statistical tests were performed using SigmaStat™ software and the criterion for statistical significance was $P<0.05$.

During mechanical and thermal stimulation the withdrawal responses from the 4 trials were averaged. The mean values from all the animals in the group were averaged and means ± SEM were calculated for each experimental condition and time point. For SNL study, Two-way repeated measures analysis of variance (RM ANOVA) followed by Holm-Sidak *post hoc* test was used for the ipsilateral versus contralateral results comparisons as the between-subject variable and time as the repeated measure, to analyze differences over time between the experimental and control paw in every group of animals. Two-way ANOVA was used to assess statistical differences at different testing time points between the experimental groups. For the PIPN study, Two-way ANOVA followed by Holm-Sidak *post hoc* test was used to assess statistical differences in PWT at different testing time points between the experimental groups. One-way ANOVA followed by Holm-Sidak *post hoc* test was used for the differences over time in blood pressure compared with the basal level.

For the western blot data analyses in the SNL study, Mann-Whitney Rank Sum Test was used for the statistical comparison of protein levels between ipsi- and contralateral sides, as well as between the SNL group and the LOS group, followed by Bonferroni correction to counteract the problem of multiple comparisons. One-way ANOVA followed by Holm-Sidak *post hoc* test was used for the statistical comparison of protein levels between the experimental groups in the PIPN study.

For immunohistochemistry, IR% ratios were averaged from 4 to 10 sections for each DRG and DR for each animal and averages were further used for statistical analysis. Wilcoxon Signed Rank test was used to compare the differences between ipsilateral and contralateral sides for each group of animals and Mann-Whitney U test was used to determine the significance of the difference between the SNL and LOS groups, the criterion for statistical significance was $P<0.05$.

For RT PCR and ELISA experiments, One-way ANOVA was followed by Holm-Sidak *post hoc* test. All values are expressed as means ± SEM. All statistical tests were performed using SigmaStat™ software.

5. RESULTS

5.1 Anti-inflammatory effect of systemic losartan treatment in the model of SNL-induced peripheral neuropathy (Publication A)

5.1.1 The effect of losartan p.o. treatment on the SNL-induced macrophage accumulation in DRGs and dorsal roots

Growing body of studies indicate the accumulation of macrophages in the DRGs corresponding to the site of the peripheral nerve injury. In this study, we used spinal nerve ligation model to evaluate the effect of systemic losartan administration on the accumulation of macrophages in L5 DRGs and dorsal roots. Analysis of the macrophage accumulation was based on the immunohistochemical evaluation of the CD68-positive cells expression in L5 DRGs and adjacent DR. Two sections of DR were compared, the one closer to the DRG (approximately 5 mm from the DRG - gDR) and the other one closer to the spinal cord (5 mm from the spinal cord - scDR). In the SNL group, we measured an enhanced accumulation of CD68-positive macrophages in the L5 DRGs ipsilateral to the injury, when compared to the contralateral DRGs (Figure 5.1. A, B and E). The area of CD68 immunopositivity (IR%) measured in the ipsilateral DRGs (2.51 ± 0.63 %) was significantly higher when compared to the contralateral side (0.08 ± 0.03 %, $P<0.05$) in the SNL group. Macrophage accumulation was also enhanced in the ipsilateral gDR of the SNL animals (0.92 ± 0.37 %, $P<0.05$), while it was very low (0.08 ± 0.02 %) in the contralateral gDR. In the scDRs, the difference in the CD68 IR% between the ipsilateral and contralateral sides was not significant (0.71 ± 0.32 % and 0.23 ± 0.09 %). Losartan *p.o.* treatment resulted in significant attenuation in the macrophage infiltration into DRGs (Figure 5.1. C, D and E). In the LOS group, CD68 IR% in the ipsilateral DRGs and gDRs was significantly lower, when compared to the SNL group (0.49 ± 0.34 %, and 0.07 ± 0.03 %, $P<0.05$). Losartan treatment also diminished any side-to-side differences between the DRGs, gDR and scDR after the SNL induction.

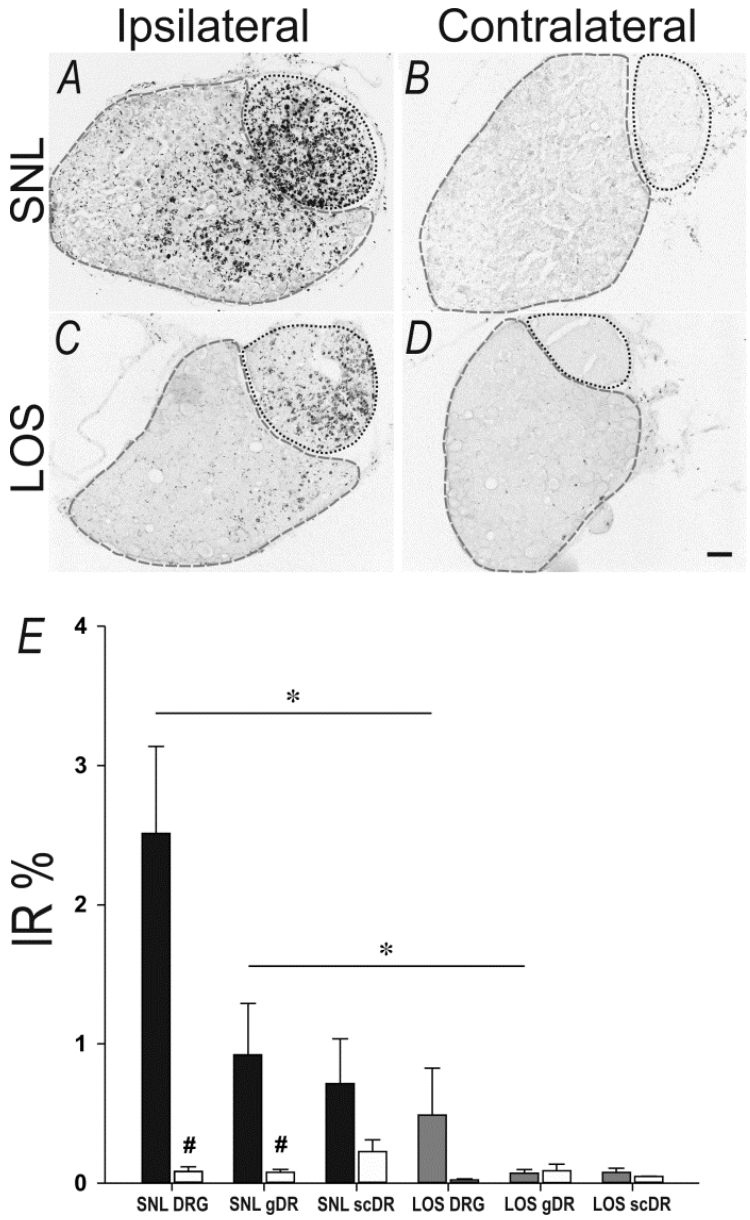

Figure 5.1. Systemic losartan treatment attenuated SNL-induced macrophage infiltration into adjacent DRG. *Representative immunohistological sections illustrate the accumulation of CD68-positive cells in L5 DRGs on the ipsilateral (**A, C**) and contralateral (**B, D**) sides in rats from the SNL (**A, B**) and LOS (**C, D**) groups. Gray dash line outlines DRG bodies and black dotted line outlines the area of dorsal roots. Scale bar equals 100 μm. (**E**) Illustrates the change in CD68-imunoreactive area (IR%) in the ipsilateral DRGs and dorsal roots adjacent to DRG (gDR) and spinal cord (scDR) from SNL group (black bars) and LOS group (grey bars) and corresponding contralateral sides (white bars). Data are presented as means ± SEM. Wilcoxon Signed Rank test was used to compare the differences*

between the ipsilateral and contralateral sides for each group of animals (# P<0.05) and Mann-Whitney U test was used to determine the significance of the difference between SNL and LOS groups (P<0.05).*

5.1.2 Effect of losartan p.o. treatment on the expression of SNL-induced pro-inflammatory markers in the spinal cord

For this study, we also used western blot analyses to evaluate the effect of systemic losartan treatment on the expression of pro-inflammatory proteins in the spinal cord of SNL-operated rats. Our results demonstrate that pro-inflammatory cytokine TNFα (Figure 5.2.A, 124.9 ± 4.8 %) and the chemokine CCL2 (Figure 5.2.C, 119.1 ± 5.8 %) levels were significantly elevated in the ipsilateral side of the lumbar SC after the SNL surgery (P<0.005 for both proteins). Protein levels for their receptors were also increased in SNL-operated rats: TNFR1 (Figure 5.2.B, 147.8 ± 10.6 %, P<0.005) and CCR2 (Figure 5.2.D, 154.3 ± 8.7 %, P<0.005) respectively. On the other side, cytokine IL6 protein levels were not significantly affected by the SNL surgery at this time point (Figure 5.2.E, 112.9 ± 6.8 %). The presence of neuroinflammatory changes were confirmed by the increase of OX42 (CD11b) protein levels in the ipsilateral side of spinal cord after the SNL (Figure 5.2.F, 130.4 ± 6.7 %, P<0.005). However, no significant changes in the astrocyte activation marker – GFAP - levels were detected (Figure 5.2.G, 101.2 ± 2.1 %). These data suggest that at the time point of measurement (the day 7 after the SNL) there was a significant microglia activation and no detectable astrogliosis in the ipsilateral spinal cord.

Losartan *p.o.* treatment (100 mg/kg) abolished the SNL-induced overexpression of pro-inflammatory markers TNFα (Figure 5.2.A, 82.3 ± 5.5 %), CCL2 (Figure 5.2.C, 119.1 ± 5.8 %) and their respective receptors TNFR1 (Figure 5.2.B, 92.5 ± 5.6 %) and CCR2 (Figure 5.2.D, 102.0 ± 7.8%) in the ipsilateral SC. Moreover, we did not observe signs of microgliosis in the LOS group, as ipsilateral OX42 levels were significantly lower than those in the SNL group and did not differ from the corresponding contralateral values (Figure 5.2.F, 100.5 ± 6.5 %).

SNL induced also a significant elevation in protein levels of AT1R in the ipsilateral SC (Figure 5.2.H, 145.8 ± 14.3 %, P<0.025), which was prevented by the losartan *p.o.* treatment (101.7 ± 8.6 %).

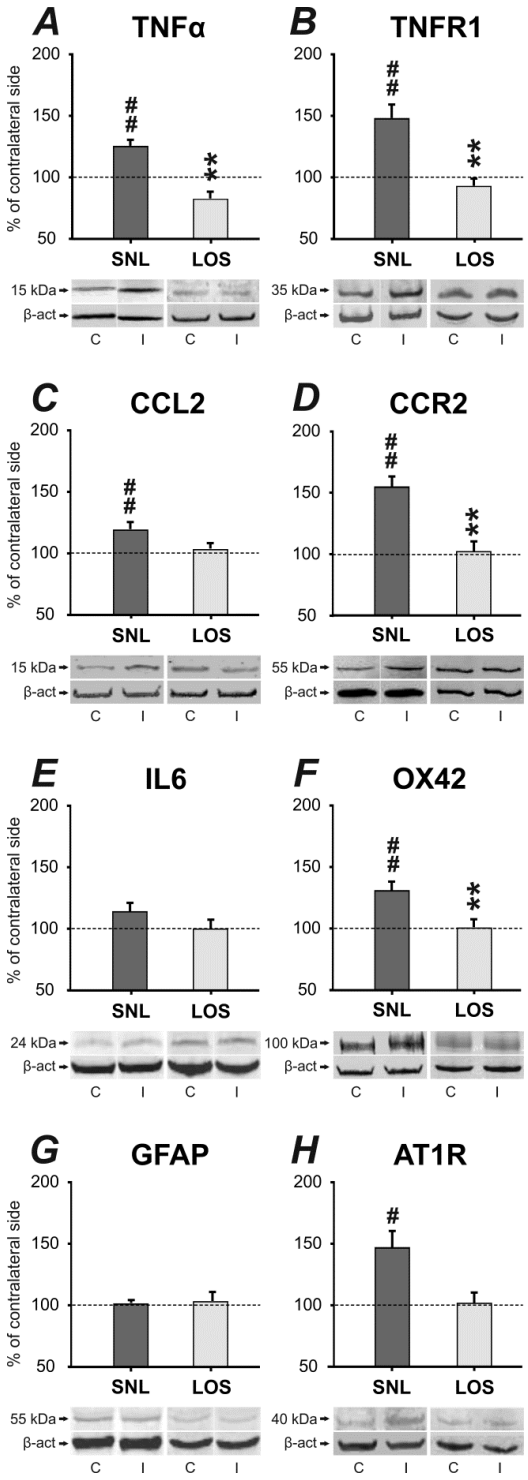

Figure 5.2. Losartan treatment prevented the SNL-induced increase in protein expression of neuroinflammatory markers in the ipsilateral spinal cords. *The samples were collected 7 days after the SNL surgery. Relative protein quantification for comparisons between the SNL-operated rats (SNL group, dark grey*

*bar) and the SNL-operated rats with losartan p.o. treatment (LOS group, light grey bar). Each value is normalized to the reference protein (β-actin) and ipsilateral spinal cord data are expressed in percent of the contralateral value for each group. Data are presented as means ± SEM. Mann-Whitney Rank Sum Test was used for the statistical comparison of protein levels between the ipsilateral (I) and contralateral (C) sides (# P<0.025; ## P<0.005), as well as between the SNL and the LOS groups (** P<0.005), followed by Bonferroni correction.*

5.2 Anti-allodynic and anti-inflammatory effects of systemic losartan treatment on paclitaxel-treated rats (Publication B)

5.2.1 Systemic losartan treatment attenuates paclitaxel-induced mechanical allodynia in rats

In this study, animals were treated with paclitaxel (5x2 mg/kg, *i.p.* injections) on 5 alternate days. Already on the day 7 after the first injection (after only 3 injections of paclitaxel) the PWT to mechanical stimuli in the PAC group significantly decreased to 85.6 ± 2.1 % (P<0.01 when compared to the VEH group, n = 30, Figure 5.3.) and remained significantly lowered on the day 10 (86.4 ± 2.6 %, P<0.001 when compared to the VEH group (n = 26)). This phase of the PIPN we consider as an **acute,** as paclitaxel treatment was still in progress. Notably, during acute phase, paclitaxel was concentrated mostly in DRGs and affected primarily DRG neurons. During the next phase (referred here as **chronic**), the PWT remained significantly decreased in the PAC group and were 84.2 ± 2.1 % and 84.5 ± 2.0 % of the CTRL value on days 14 and 21 respectively (P<0.001, when compared to the VEH group). Another experimental group of rats received systemic treatment with losartan (100 mg/kg/day) simultaneously with paclitaxel injections (from the day 1 until the end of the experiment, LOS group, n = 28). The behavioral changes observed in the LOS group seem to be two-phasic. During the acute phase of the PIPN, the PWL to mechanical stimuli tend to decrease in the same manner as in the PAC group (92.3 ± 2.5 % of the CTRL value at the day 10). This decrease, however, was not significantly different from the VEH group value. The exception is an early measurement on the day 7,

when the PWT did not differ from the VEH group values, but were significantly higher than in the PAC group (97.3 ± 2.1 %, P<0.001). During the chronic phase of the PIPN, we observed a strong preventive effect of losartan treatment. On days 14 and 21 after the first injection, the LOS group exhibited significantly higher levels of the PWL, when compare with the PAC group (99.7 ± 2.1 % and 96.5 ± 2.0 % respectively, P<0.001 for both values).

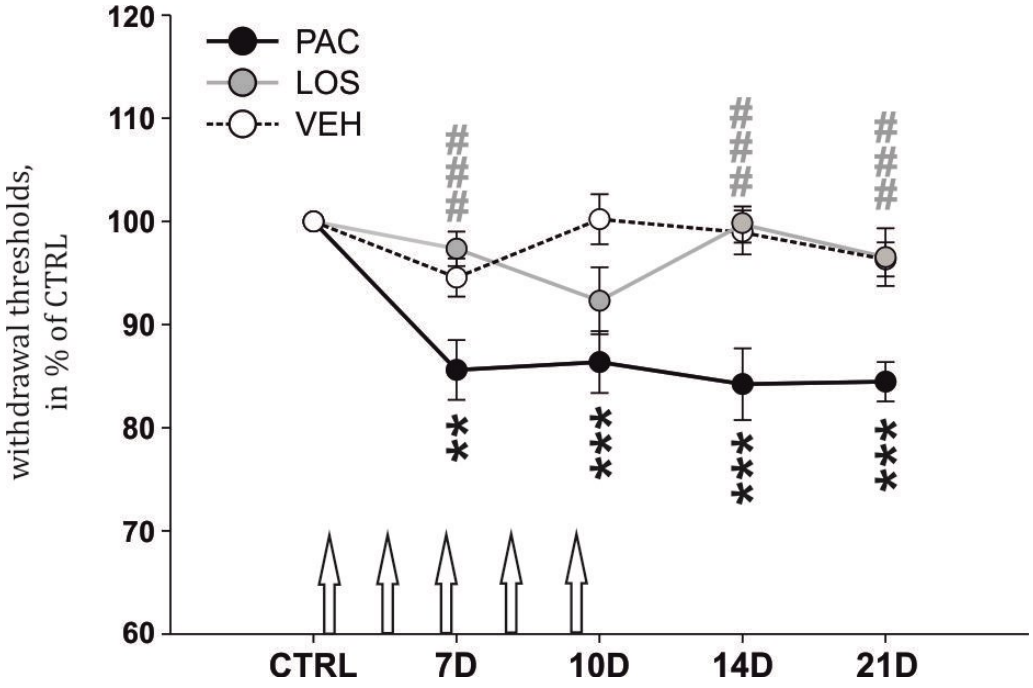

Figure 5.3. Systemic treatment attenuated the development of mechanical allodynia, induced by repeated paclitaxel injections. *Experimental values are normalized to the CTRL value, assessed for each group before the start of the treatment. Data are presented as means ± SEM. Two-way ANOVA followed by Holm-Sidak post hoc test was used to assess the significance of the statistical differences in the PWT at different testing time points between the experimental groups. Asterisks indicate the significance level of the significance when compared to the VEH group (** P≤0.01, *** P≤0.001). Pound signs were used to depict significant differences when compared to the PAC group (### P≤0.001).*

5.2.2 Losartan has moderate effect on PAC-induced neuroinflammatory changes in DRGs and SCDH during the acute phase of the PIPN

In this study, we define first 10 days from the beginning of the treatment as an acute phase of the PIPN. The values of inflammatory proteins expression and specific mRNA levels measured on the day 10 suggest the neuroinflammatory changes **predominantly in the DRGs**. Our results demonstrate a significant elevation of satellite glial cells (SGC) activation marker GFAP protein levels ($P < 0.05$ vs. VEH group, n = 7 and 5 respectively, Figure 5.4.A), as well as key inflammatory chemokine CCL2 ($P < 0.01$, n = 7 and 8). In our experiments, we observed also the presence of macrophage M1-like cell line markers in DRGs of paclitaxel-treated animals. We measured the elevated levels of CD11b ($P < 0.001$, n = 4) and CD68 ($P < 0.01$, n = 5) proteins along with mRNA levels for CD68 ($P < 0.001$, n = 5, Figure 5.5.A) and TNFα ($P < 0.05$) in the PAC group, when compared to the VEH group of animals. The presence of activated macrophages in the PAC group was also confirmed by increased protein levels of TNFα ($P < 0.05$, n = 7, Figure 5.4.A), as well as of its specific receptor TNFR1 ($P < 0.05$, n = 5). Losartan treatment attenuated the activation of SGCs, as GFAP protein levels in the LOS group were significantly lower than in the PAC group ($P < 0.01$, n = 7). The elevation in CCL2 chemokine expression was also prevented, as its protein levels in the LOS group did not differ from the VEH group. In DRGs of losartan treated rats, we did not observe the elevation of M1 macrophage markers such as their main secreted cytokine TNFα (n = 6), neither its mRNA, nor its receptor TNFR1 (n = 4), or phagocytosis marker CD68 (n = 5). Moreover, losartan treatment significantly attenuated paclitaxel-induced CD68 mRNA expression ($P < 0.001$ when compared with PAC group, n = 6, Figure 5.5.A). Surprisingly, we still observed a significant upregulation of CD11b protein expression in the LOS group when compared with control group ($P < 0.001$, n = 4, Figure 5.4.A).

In the spinal cord dorsal horn, we measured an increase in CD11b protein levels in the PAC group (Figure 5.4.B), which was blocked with losartan treatment ($P < 0.05$, n = 7 in the PAC group and n = 6 for the LOS group). Regarding mRNA expression, we observed a significant drop in iNOS mRNA expression in the PAC group ($P < 0.01$, n = 5, Figure 5.5.B), while in the LOS group it was not significantly different from the VEH group. We observed no significant changes in mRNA or protein levels of other inflammatory proteins in the spinal cord at this time point.

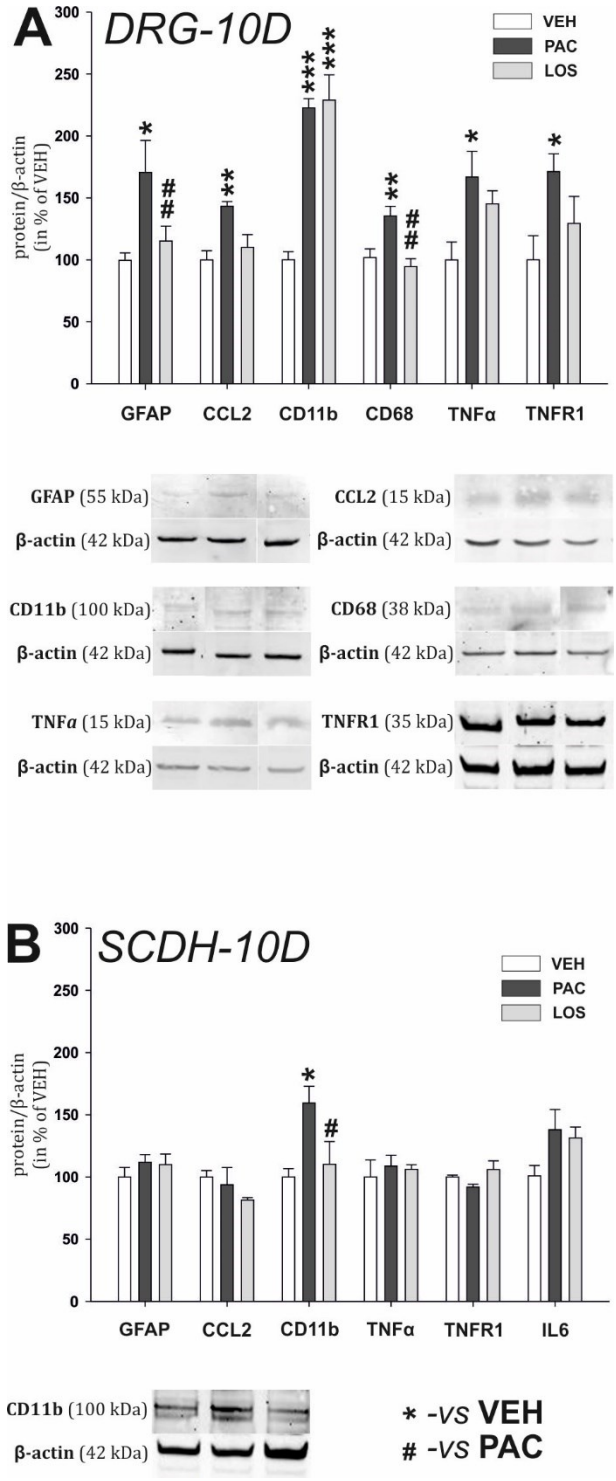

Figure 5.4. Relative protein expression levels in DRGs (A) and SCDH (B) in experimental animals during the acute phase of the PIPN (on the day 10 after the first PAC injection). *Representative western blot captures for significantly*

*affected proteins are presented below the corresponding graphs. Data are normalized to β-actin, standardized to the value in the VEH group and are presented as means ± SEM. One-way ANOVA followed by Holm-Sidak post hoc test was used for the statistical evaluation. Asterisks indicate the significance of the comparisons to the VEH group (*P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001). Pound signs were used to depict the significant differences when compared to the PAC group (#P ≤ 0.05, ##P ≤ 0.01).*

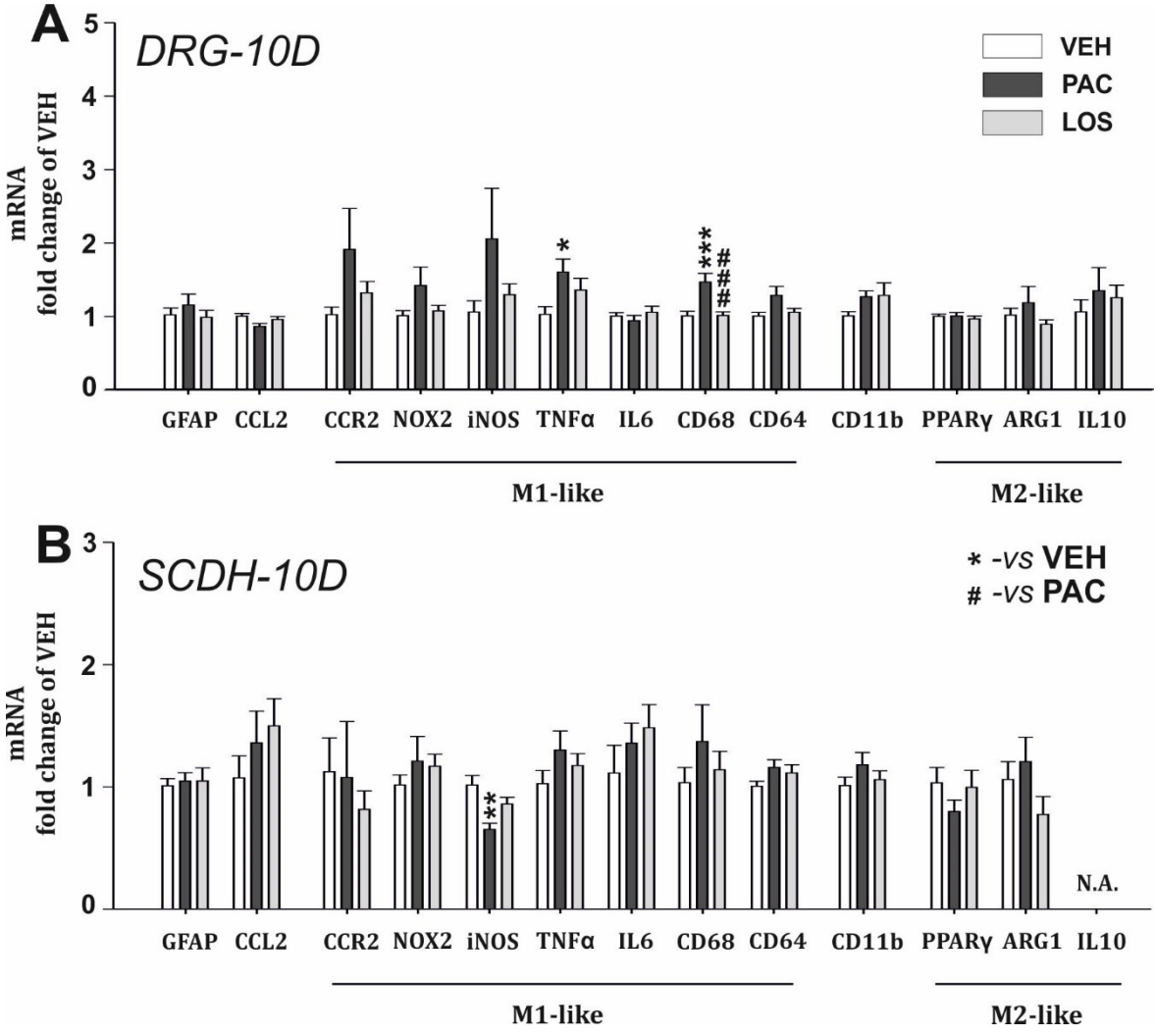

Figure 5.5. Relative mRNA levels in DRGs (A) and SCDH (B), measured during the acute phase of the PIPN (at the day 10 after the first PAC injection). *VEH group (n = 6), PAC group (n = 5), and LOS group (n = 6). One-way ANOVA followed by Holm-Sidak post hoc test was used for the statistical evaluation. Asterisks*

*indicate the significance of the comparisons to VEH group (*P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001). Pound signs were used to depict the significant differences when compared to the PAC group (###P ≤ 0.001).*

5.2.3 Losartan attenuates the signs of PAC-induced neuroinflammation in DRGs and SCDH during chronic phase

In order to investigate the neuroinflammatory changes during the chronic phase of the PIPN, we performed measurements of protein and mRNA levels at the day 21 after the start of the paclitaxel treatment. **In DRGs** from paclitaxel-treated animals, we measured elevated levels of pro-inflammatory proteins: chemokine CCL2 ($P < 0.001$, $n = 8$), cytokine IL6 ($P < 0.01$, $n = 8$) and macrophage markers CD11b ($P < 0.05$, $n = 6$) and CD68 ($P < 0.01$, $n = 8$, Figure 5.6.A). We also measured a significant increase in mRNA levels for specific M1 macrophage markers - CCR2 ($P < 0.01$), NOX2 ($P < 0.001$), iNOS ($P < 0.05$), CD64 ($P < 0.001$), CD68 ($P < 0.001$) and for CD11b ($P < 0.01$), when compared to the VEH group. On the other side, the values measured in the LOS group indicate a significant decrease in protein expression of CCL2 ($P < 0.01$, $n = 8$) and prevention from IL6 overexpression when compared with PAC group. While CD11b levels were still significantly elevated ($P < 0.01$ vs. VEH group, $n = 6$), the paclitaxel-induced protein overexpression of phagocytic marker CD68 was significantly prevented by losartan treatment ($P < 0.01$, $n = 7$). Regarding mRNA levels in DRGs, we observed a preventive effect of losartan administration on the upregulation of principal M1 macrophage markers when compared to the PAC group - NOX2 ($P < 0.01$, Figure 5.7.A), CD11b ($P < 0.05$) and CD68 ($P < 0.05$). In contrast to the PAC group, in the LOS group mRNA levels for CCR2, iNOS and CD64 did not differ from the VEH group values. In addition to this, we measured a significant upregulation of the main M2 macrophage anti-inflammatory product - IL10 mRNA expression ($P < 0.05$ vs. VEH and $P < 0.01$ vs. PAC groups), as well as ARG1 mRNA ($P < 0.05$ vs. PAC and VEH groups) along with its protein product Arginase 1 ($P < 0.05$ vs. VEH, $n = 6$, Figure 5.8.). Notably, PPARγ mRNA expression was also significantly increased in rats with losartan treatment ($P < 0.01$ vs. VEH group). **In the spinal cord dorsal horn**, we observed no signs of microglial activation (no significant increase in CD11b protein expression), but robust astrocytosis, reflected in significant elevation of GFAP protein levels ($P < 0.001$, $n = 7$, Figure 5.6.B) in the PAC group. Moreover, the protein levels of the key inflammatory modulators, CCL2

and TNFα, were also significantly increased ($P < 0.01$, $n = 6$; and $P < 0.001$, $n = 8$, respectively). Losartan treatment abolished paclitaxel-induced upregulation of protein expression for GFAP ($P < 0.001$, $n = 7$) and for the main chemokine produced by inflamed astrocytes, CCL2 ($P < 0.05$, $n = 7$). TNFα and IL6 protein levels were also affected by losartan treatment and significantly decreased when compared to the PAC group ($P < 0.001$, $n = 8$; and $P < 0.05$, $n = 8$ respectively). However, we did not detect any changes in the mRNA expression for these proteins at this time point (Figure 5.7.B).

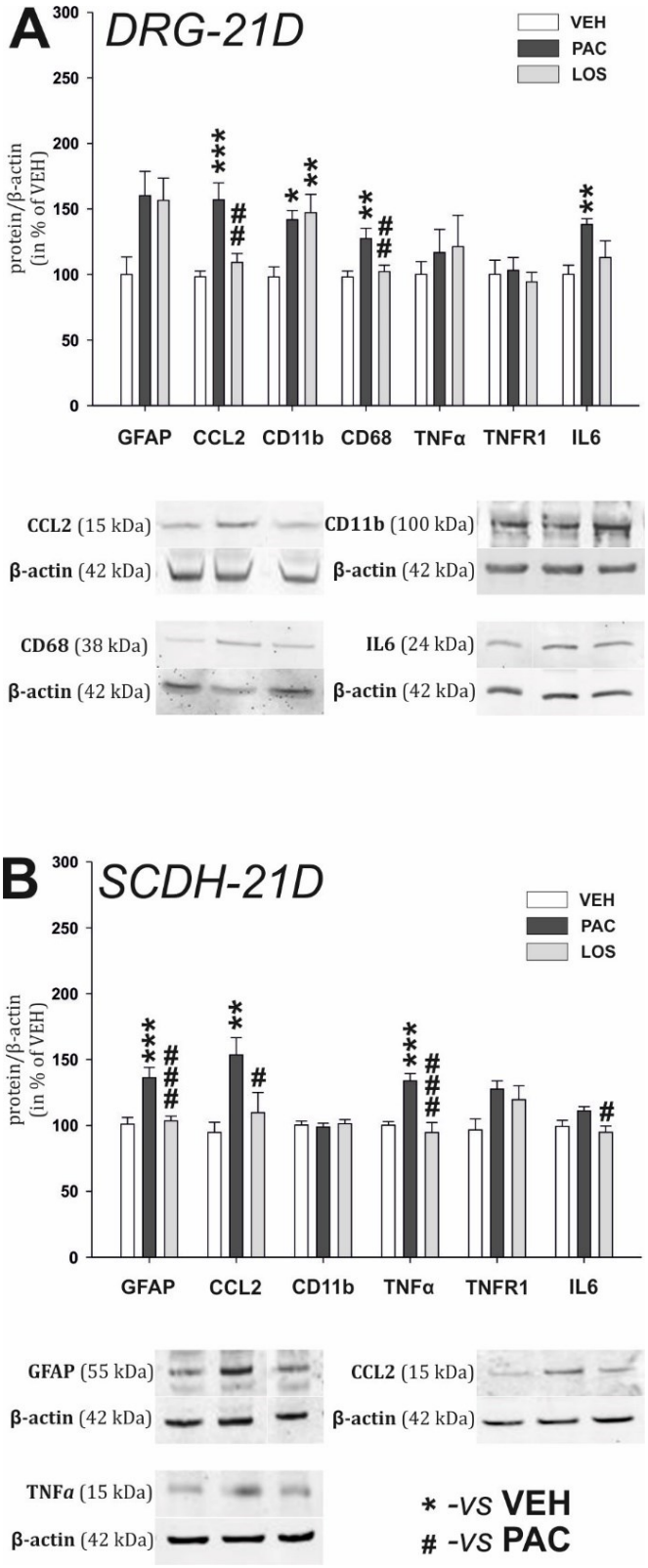

Figure 5.6. Relative protein expression levels in DRGs (A) and SCDH (B) in experimental animals during the chronic phase of the PIPN (on the day 21). *Representative western blot captures for significantly affected proteins are presented below the corresponding graphs. Data are normalized to β-actin, standardized to the*

*value in the VEH group and are presented as means ± SEM. One-way ANOVA followed by Holm-Sidak post hoc test was used for the statistical evaluation. Asterisks indicate the significance of the comparisons to the VEH group (*P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001). Pound signs were used to depict the significant differences when compared to the PAC group (#P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001).*

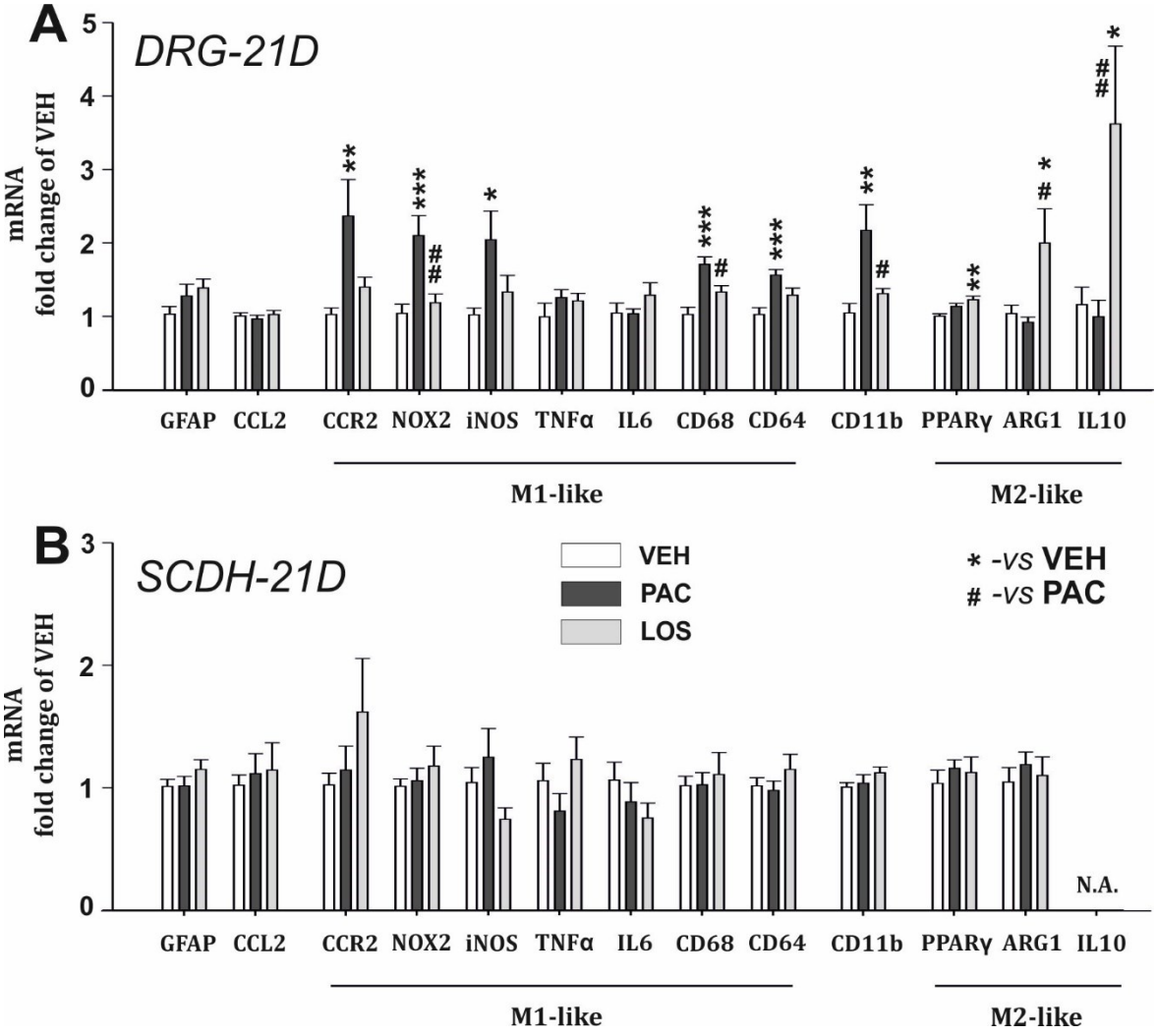

Figure 5.7. Relative mRNA levels in DRGs (A) and SCDH (B), measured during the chronic phase of the PIPN (on the day 21 after the first PAC injection). *One-way ANOVA followed by Holm-Sidak post hoc test was used for the statistical evaluation. Asterisks indicate the significance of the comparisons to the VEH group (*P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001). Pound signs were used to depict the significant differences when compared to the PAC group (#P ≤ 0.05, ##P ≤ 0.01).*

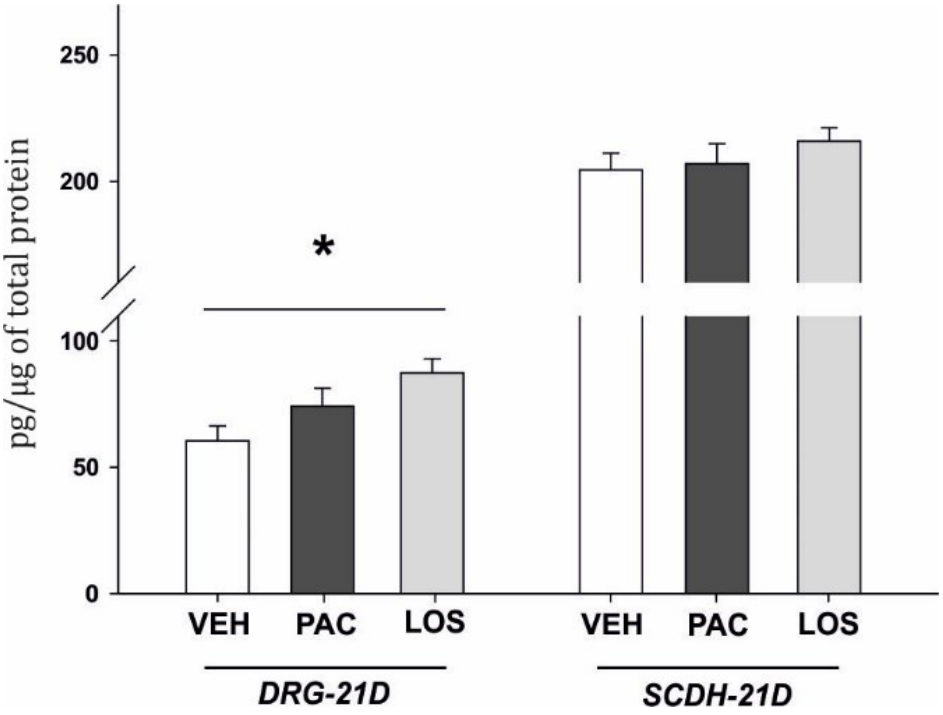

Figure 5.8. ELISA measurements of Arginase 1protein levels in DRGs and SCDH on the day 21 of the PIPN. *Data are presented as means ± SEM. One-way ANOVA followed by Holm-Sidak post hoc test was used for the statistical comparison. Asterisk indicate the significance of the comparison to VEH group (*P ≤ 0.05).*

5.3 The effect of paclitaxel treatment on neuronal activation in the SCDH (Publication C)

In order to test the effect of paclitaxel (PAC) treatment on the neuronal activation in superficial spinal cord, we measured the number of c-Fos-positive cells in the lumbar spinal cord slices. In our experiments, the incubation for 1 hour with 100 nM PAC significantly increased the number of c-Fos immunoreactive neurons in laminae I/II of the spinal cord dorsal horn (Figure 5.9.). The effect of incubation with PAC was observed not only on the number of c-Fos positive neurons, but also on the intensity of the staining.

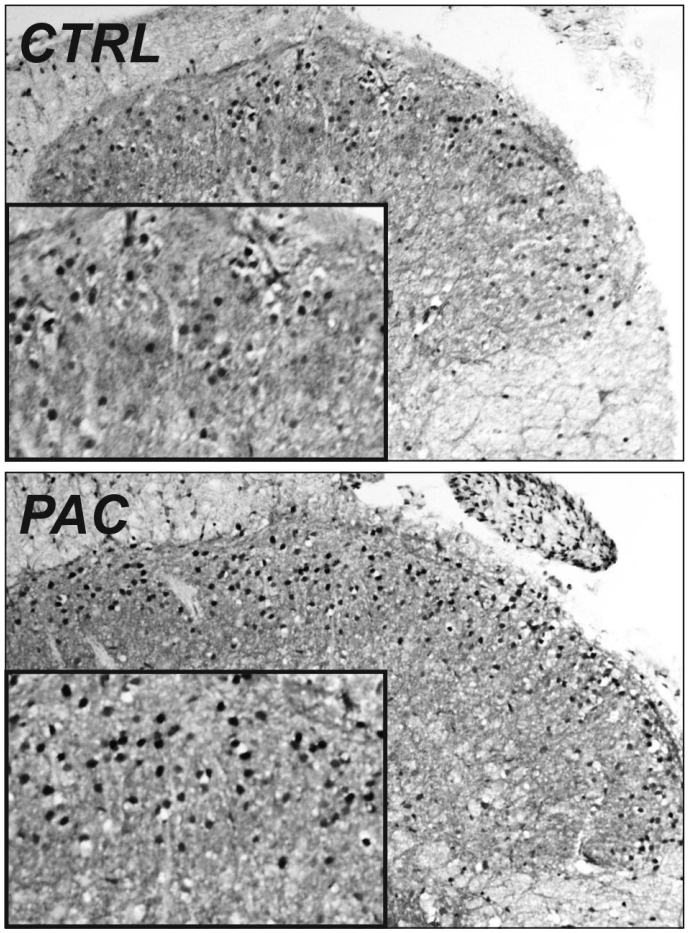

Figure 5.9. Paclitaxel-induced upregulation of c-Fos expression in neuronal nuclei in the superficial area of spinal cord dorsal horn. *Acute spinal cord slices were incubated either with vehicle (CTRL) or with 100 nM paclitaxel (PAC). Inserts demonstrate c-Fos positive neuronal nuclei in the same slices at higher magnification.*

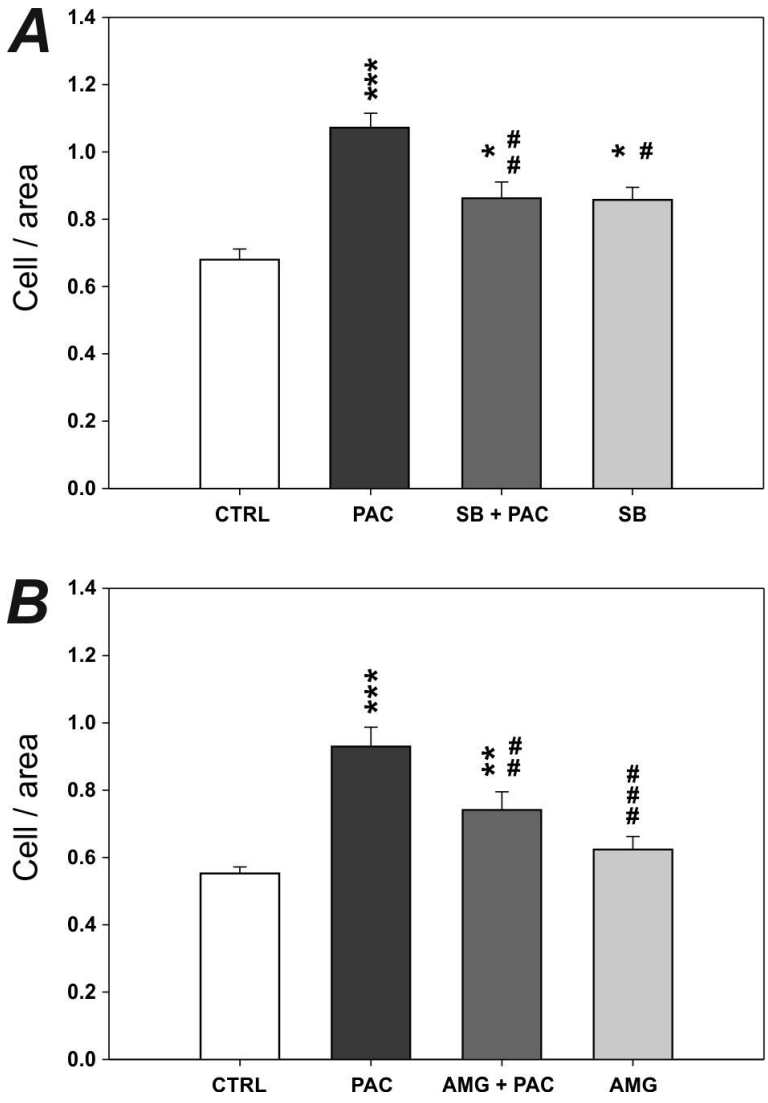

Figure 5.10. Treatment with TRPV1 antagonist attenuates PAC-induced neuronal activation in the SCDH. *The expression of c-Fos-positive cells was measured in the superficial spinal cord dorsal horn in control group (CTRL), after the paclitaxel treatment (PAC), with TRPV1 antagonists added (SB+PAC; AMG+PAC), and TRPV1 antagonists only (SB; AMG). Data are presented as means ± SEM. Asterisks indicate significant difference from the control group (One-way ANOVA, Holm-Sidak post hoc test, *P<0.05; **P<0.01; ***P<0.001). Pound signs indicate significant difference from the PAC group (One-way ANOVA, Holm-Sidak post hoc test, # P<0.05; ## P<0.01; ### P<0.001).*

Preincubation with TRPV1 receptor antagonist SB366791 (10uM for 10 min prior to the paclitaxel) suppressed the paclitaxel-induced c-Fos protein expression (Figure 5.10.A, $P < 0.01$). However, both experimental groups incubated with SB336791 (SB+PAC

and SB) demonstrated a significant increase in the number of c-Fos immunoreactive neurons when compared to the CTRL group (Figure 5.10.A, $P<0.05$ for both groups). Thus, preincubation with SB366791 suppressed c-Fos induction by paclitaxel treatment, but induced minor elevation of c-Fos immunoreactivity on its own when compared with the CTRL group. In order to compare SB366791 with another TRPV1 receptor antagonist, we examined the effect of AMG9810 on paclitaxel-induced c-Fos immunoreactivity in dorsal horn neurons. Similarly to SB366791, preincubation with AMG9810 (10uM for 10 min prior to paclitaxel) attenuated paclitaxel-induced c-Fos expression ($P<0.01$, AMG+PAC, Figure 5.10.B). Additionally, the AMG9810 treatment alone did not show any significant changes from the CTRL group in the c-Fos expression.

5.4 The involvement of TRPV1 signaling in the development of acute CCL2-induced painful hypersensitivity (Publication D)

5.4.1 CCL2-induced thermal and mechanical sensitivity is abolished by TRPV1 antagonist SB366791 treatment in rats

In order to evaluate the involvement of TRPV1 receptors in the development of CCL2-induced hyperalgesia, we treated adult Wistar rats with TRPV1 receptor antagonist and/or CCL2 via intrathecal application. The responsiveness to thermal (PWL) and to mechanical stimuli (PWT) was measured after the *i.t.* application of CCL2 (1.4 mg per rat, n = 9) using an implanted lumbosacral catheter. Intrathecal saline application had no effect on either the thermal or the mechanical sensitivity, thus, it was used as a control (n = 6, Figure 5.1.A and B). On the contrary, CCL2 *i.t.* application induced a significant decrease in both the PWL and the PWT already 1 hour after (79.9 ± 3.4 % and 76.1 ± 4.9 % respectively, Figure 5.11.A and B), when compared to saline treatment (103.4 ± 2.3 % for PWL and 95.7 ± 2.7 % for PWT). These data confirmed the presence of thermal hyperalgesia and mechanical allodynia after the intrathecal application of CCL2. In the next experimental group, the TRPV1 receptor antagonist SB366791 (0.43 mg, n = 7) was applied intrathecally 15 min before CCL2 (1.4 mg) application. SB366791 pretreatment

virtually abolished the development of CCL2-induced thermal hypersensitivity (99.0 ± 4.9 % at 1h, Figure 5.11.A), and the PWL did not differ from those observed following saline treatment. In contrast, TRPV1 antagonist pretreatment failed to prevent the establishment of mechanical hypersensitivity, as the SB366791- treated group showed a similar significant PWT decrease at 1 hour after the CCL2 application (75.0 ± 8.9 % at 1h, Figure 5.11.B).

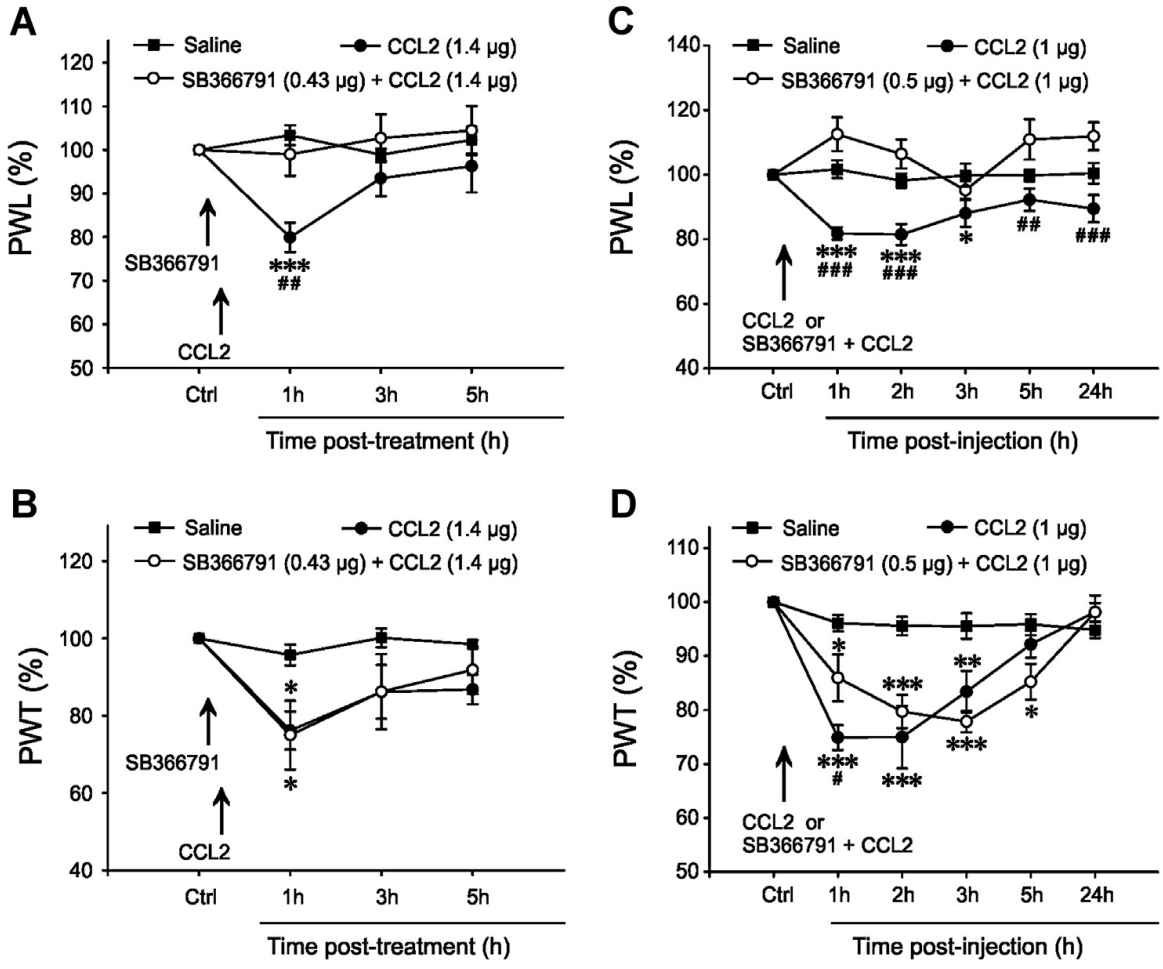

Figure 5.11. TRPV1 receptor antagonist diminished thermal hyperalgesia induced by the chemokine CCL2. *(A) The intrathecal application of CCL2 using catheter implantation for drug delivery decreased the PWL to thermal stimuli, which was prevented by pretreatment with the specific TRPV1 receptor antagonist SB366791. (B) I.t. CCL2 application using a lumbosacral catheter decreased the PWT to mechanical stimuli. SB366791 pretreatment did not affect the PWT change induced by CCL2. (C) A single acute intrathecal injection of CCL2*

*induced thermal hyperalgesia, which was prevented by the co-injection of SB366791 and CCL2. (D) An i.t. injection of CCL2 induced mechanical hypersensitivity. The i.t. co-injection of SB366791 + CCL2 diminished the pronociceptive CCL2-induced effect at 1 h after the co-injection, while it failed to prevent the induction of mechanical allodynia at other time points. Saline administration using an i.t. catheter (A, B) or using an i.t. injection (C, D) did not significantly change the control PWL and PWT values. Two-way ANOVA with the Bonferroni post hoc test was used for statistical analyses; *P < 0.05, **P < 0.01, ***P < 0.001 versus saline administration; #P < 0.05, ##P < 0.01, ###P < 0.001 versus SB366791 and CCL2 administration.*

Our experiments using lumbosacral catheters demonstrated the CCL2-induced hyperalgesia only at one time point. However, previous studies indicated the induction of prolonged hypersensitivity lasting up to several days after a single CCL2 intrathecal or intraspinal injection (Dansereau et al., 2008, Thacker et al., 2009). Considering the fact that the catheter implantation may induce local tissue inflammation, which could be associated with changes at the spinal cord level, we decided to perform analogic behavioral experiments with distinct route of administration. For the second set of behavioral experiments in this study, we administered the CCL2 or SB366791+CCL2 combination via single intrathecal injection in rats (n = 7 for both groups, Figure 5.11.C and D). We also assessed the PWL and PWT at several additional time points (at 2h and 24h after the drug injection). An *i.t.* injection of saline did not induce any change in the PWL or PWT compared to the pretreatment values in the same manner as in the previous set of experiments (n = 7, Figure 5.11.C and D). CCL2 (1 mg) *i.t.* injection significantly decreased the thresholds for both thermal and mechanical stimuli from 1 h to 3 h after the injection, compared to saline application (Figure 5.11C and D). The most pronounced CCL2-induced pronociceptive effect was plateau-shaped between 1 h and 2 h, subsided at 3 h and later returned to the values obtained in the saline-treated group at 5 h and 24 h post injection. TRPV1 receptor antagonist SB366791 (0.5 mg) was also injected intrathecally with the chemokine CCL2 (1 mg). The combined *i.t.* injection of SB366791 + CCL2 abolished the decrease in PWL (106.4 ± 4.5 % in SB366791 + CCL2 group vs 81.8 ± 1.9 % in SB366791 group at 1 h, P < 0.001, Figure 5.11.C). Moreover, the threshold for the PWL was slightly increased (without statistical significance) after SB366791 + CCL2 injection at 1 h, 2 h, 5 h and 24 h compared to saline administration. We also observed a significant difference between the SB366791 + CCL2-treated and the CCL2-treated groups in the PWT. The *i.t.* injection of SB366791 + CCL2 combination diminished the mechanical

hypersensitivity at 1 h after the injection (86.0 ± 4.3 %), when compared to the treatment with CCL2 alone (74.9 ± 5.8 %). Notably, this decrease in the PWT was still significantly different from the PWT in the saline-treated group (96.1 ± 1.5 %, Figure 5.11.D). Thus, co-injection with SB366791 did not significantly attenuate the CCL2-induced increased mechanical sensitivity in rats. Our results indicate an important role of spinal TRPV1 receptor activation in the mechanisms of thermal hypersensitivity induced by the intrathecal administration of the chemokine CCL2.

5.4.2 The effect of TRPV1 receptor antagonist on the CCL2-induced activation of pERK in the SCDH

In this study, we decided to investigate the role of spinal TRPV1 receptors in the mechanism of activation of ERK by the chemokine CCL2. In order to fulfill this aim, we incubated acute spinal cord slices with CCL2 or with SB366791 and CCL2. In this set of experiments, CCL2 induced an increase in the number of pERK-positive cells labeled in the superficial spinal cord dorsal horn to 139 ± 8% when compared to the control slices ($n = 5$, $P < 0.01$; Figure 5.12.A and B). The incubation with the combination of SB366791 and CCL2 did not increase the number of pERK-positive cells significantly, which reached only 114 ± 7% of the control value ($n = 5$, $P > 0.05$; Figure 5.12.C). Notably, the difference between the treatment with CCL2 alone and the SB366791/CCL2 treatment was statistically significant ($P < 0.05$; Figure 5.12.D). Our immunohistochemical analysis indicates that the increased pERK labeling induced by CCL2 was diminished by TRPV1 receptor antagonist treatment in the superficial SCDH.

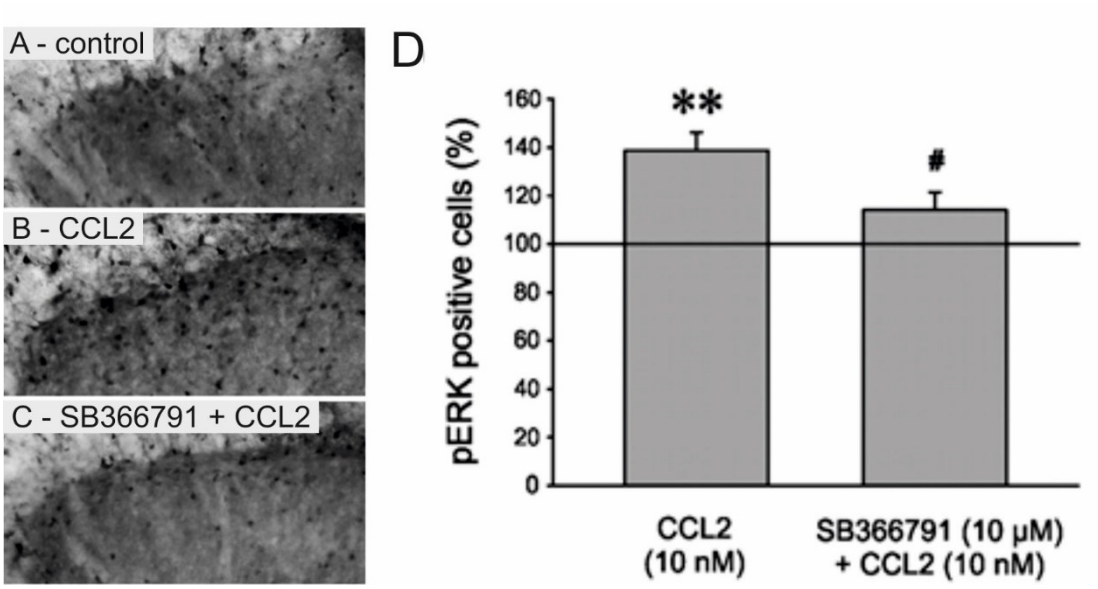

Figure 5.12. The expression of pERK-positive cells in the superficial SCDH in CCL2 or SB366791/CCL2 treated slices. *(A) Basal pERK labeling in the control spinal cord slice (200 mm thick). (B) Spinal cord slice incubation with CCL2 (10 nM) for 10 min increased the number of pERK-positive cells. (C) Preincubation of the slice with SB366791 (10 mM) for 5 min before adding CCL2 (10 nM, 10 min) decreased the CCL2-induced pERK labeling. (D) Summarized results show significant increase of pERK positive cells after CCL2 treatment (**P < 0.01). This increase was prevented with preincubation of the slices with TRPV1 antagonist SB366791 (#P < 0.05 versus CCL2). One-way ANOVA followed by the Student-Newman-Keuls test was used for statistical analyses.*

6. DISCUSSION

6.1 The effect of losartan treatment on the development of peripheral neuropathy after peripheral nerve injury

This study explores the anti-hyperalgesic and anti-inflammatory effect of systemic losartan treatment in the model of spinal nerve injury. Behavioral, molecular and IHC methods were used to evaluate the effect of losartan treatment on the development of neuroinflammatory changes in DRGs and spinal cord of SNL-operated rats. It covers the aim 1 (Publication A, see in the Appendix section).

In the present study, we confirmed that SNL induced increased sensitivity to thermal and mechanical stimuli, as well as macrophage infiltration to affected DRG and dorsal roots. The SNL-induced changes in the expression of several pro-inflammatory proteins in ipsilateral spinal cord confirmed the presence of neuroinflammatory changes. A significant reduction of the mechanical threshold (PWT) was present from the day 1 and reached its maximum at the day 9. In a similar manner, the heat response thresholds (PWL) were markedly reduced already from the day 1, exhibiting its lowest value during the second week after the SNL induction (Publication A). Our results are in accordance with the data obtained by Kim and Chung, which show the development of thermal hyperalgesia and mechanical allodynia within 24 - 48 hours, with a peak at 7-10 days after surgery and sustained throughout more than 45 days (Kim and Chung, 1992). In addition, similar to the results described by Kim and Chung, the signs of allodynia and hyperalgesia developed also on the contralateral to the injury side. However, the PWL and PWT on the contralateral side were less pronounced and the onset time of the changes was slower compared to those on the ipsilateral side.

The losartan administration both *p.o.* and *i.t.* caused a significant reduction of the SNL induced thermal hypersensitivity, but surprisingly it failed to prevent the mechanical allodynia (Publication A). On the other side, our Western blot experiments revealed a preventive effect of losartan treatment in SNL-induced CCL2 upregulation. Since CCL2 overexpression is commonly associated with mechanical hypersensitivity (Abbadie et al., 2003), and in particular CCL2 produced by DRG primary neurons (Jeon et al., 2009), we hypothesized that the different origin of CCL2 in the spinal cord and DRGs may at least

partly explain the results of our behavioral study. At the DRG level, primary nociceptors constitute the main source of CCL2; at the spinal cord level, it appears that glial cells are major players in CCL2 production (Gao et al., 2009). Our data may reflect the fact that losartan treatment is more effective in the prevention of glial CCL2 upregulation by targeting glial functions, while it appears to be less effective to influence the neuronal CCL2 production. Moreover, although macrophage infiltration into the affected DRGs was successfully attenuated by losartan treatment, it was not blocked completely. Even minor occurrence of invaded macrophages at the inflammatory site suggests chemotactic events, thus, the presence of chemoattractant substances like CCL2 in DRGs despite the losartan treatment. In addition, it is more likely that different regulatory pathways modulate thermal hyperalgesia and mechanical allodynia development (Meller and Gebhart, 1994). Hence, different effect of losartan treatment on the inhibition of the mechanisms involved in the development of thermal hyperalgesia and mechanical allodynia cannot be excluded.

The efficacy of the losartan treatment depends on its ability to penetrate the blood-brain barrier. Pharmacokinetic studies confirmed that losartan *p.o.* administration leads to satisfactory, but not complete inhibition of centrally mediated actions of angiotensin II, if the administered dose is high enough (30-100 mg/kg) (Culman et al., 1999). It is important to emphasize, that in our study we used relatively high dose of losartan for *p.o.* treatment (100 mg/kg/day) that should lead to its effective levels in the spinal cord. Moreover, *i.t.* losartan application via lumbosacral catheter induced comparable to the *p.o.* application changes in the behavioral experiments. This suggests that the main target for the treatment effect was in the spinal cord and not in the peripheral tissues. The experimental group with the *i.t.* losartan application was not used for the western blot experiments, as the intrathecal catheter could induce unspecific local changes.

The primary target of losartan, AT1R, has been initially characterized as a receptor member of RAS for its involvement in blood pressure, vasoconstriction, sodium uptake and potassium excretion in mammals' regulatory systems (Allen et al., 2000), where it is activated by Ang II. It was important to verify whether the changes in the blood pressure do not interact with the behavioral measurements (El Bitar et al., 2014). In our control experiments, we tested the effect of losartan *p.o.* treatment on the blood pressure and demonstrated a minimal effect on the behavioral responses to thermal and mechanical stimulation. It has been described that several AT1R inhibitors exhibited neuroprotective activities at very low concentrations, which were not able to affect blood pressure in normotensive rodents (Saavedra, 2012). This effect was confirmed for several sartans. Telmisartan protects cognition in normotensive rats injected with amyloid-β at very low

dose without affecting blood pressure (Tsukuda et al., 2009). In addition, candesartan at low dose as well, protects normotensive mice from secondary brain damage and improves functional recovery after brain traumatic injury (Timaru-Kast et al., 2012). These lines of evidence, in addition with our data, suggest that the neuroprotective effects of AT1R blockers are more likely independent from the blood pressure regulation function.

Since behavioral tests showed the biggest changes during the second week after the SNL, when also significant effect of losartan treatment was present, we decided to perform protein quantification of the spinal cord samples, as well as immunohistochemical labeling of invaded macrophages in DRGs and DRs, collected at the day 7 after the SNL. Western blot analyses revealed expected overexpression of OX42 (CD11b), a microglial marker, indicating the activation state of these cells. The protein levels of GFAP, an astrocytic differentiation marker, remained unchanged suggesting the absence of astrogliosis at this time point. Both microglia and astrocytes play a major role in the development of behavioral hypersensitivity in animal models of neuropathic pain, notably through the production and secretion of immune mediators (Winkelstein et al., 2001). However, it is now admitted that in chronic neuropathic process, microglia and astrocytes act at different time points. Indeed, microglial cells are activated first and are involved in the initiation phase while astroglia mediates predominantly the maintenance of neuropathic pain. We may consider that the time point of our measurement still corresponds to the initiation phase, before the occurrence of astrogliosis. This difference in the activation of glial cells was described before. Tanga and colleagues (Tanga et al., 2004) showed an early upregulation of integrin alpha M (also known as OX42 or CD11b) transcripts in lumbar SCDH after L5 nerve transection in rats, while GFAP mRNA increase was delayed. In a same manner, in a CCI mouse model, Mika and colleagues (Mika et al., 2009) found a significant increase of protein levels of OX42, but no change of GFAP in SCDH at the experimental day 7. Taken together, it is more likely that astrogliosis occurs later and sustains; meanwhile microglia activation decreases. This may constitute an explanation for the lack of astroglial activation in our study. However, other studies revealed an astrocyte activation during the development phase of neuropathy in SNL model combined with transection on the distal side of the ligation (Park et al., 2012), or an upregulation of OX42 lasting during the maintenance phase after L5 nerve transection (Obata et al., 2010). The occurrence of microgliosis and the absence of astrocyte activation at the day 7 correspond well with the increased protein levels for TNFα and CCL2 and their respective receptors TNFR1 and CCR2 in our experiments. These findings suggest

that at this time point, microglia represents the primary source of proinflammatory mediators in the spinal cord, as it was described before (Mika et al., 2013).

We observed a significant abolishment in the SNL-induced expression CCL2, CCR2, TNFα and TNFR1 in the losartan-treated group. These data suggest that losartan treatment attenuated neuroinflammatory changes on the side of the peripheral nerve injury. While it was suggested that pro-inflammatory mediators may diffuse to the contralateral side spreading thus the inflammation at this level (Koltzenburg et al., 1999, Jancalek et al., 2011), our results confirm strong unilateral manner of pro-inflammatory protein expression in SNL group.

IL6 cytokine is intensively described as a major pro-inflammatory mediator after the neural tissue injury, since IL6-deficient mice do not develop neuropathic behavior after chronic constrictive injury (Murphy et al., 1999). IL6 involvement in neuroinflammation was shown in different experimental models, such as spinal cord injury (Okada et al., 2004) or spinal nerve ligation (Arruda et al., 1998). In our study, we did not see any significant SNL-induced changes in IL6 expression in the ipsilateral spinal cord when compared with contralateral at the day 7. Several lines of evidence may be advanced to explain our results: (i) unilateral SNL in rats induced bilateral IL6 overexpression, accompanied by bilateral allodynia, due to cytokine diffusion after the release from the lesion site to the contralateral side (Dubovy et al., 2013). We cannot exclude this hypothesis in our study and the lack of significant increase of IL6 levels in the ipsilateral side may be a result of a diffusion phenomenon, leading to a secondary increase in the contralateral side. In accordance with this hypothesis, we observed a significant decrease in PWL and PWT in contralateral sides of SNL-operated rats (Publication A). (ii) In a more relevant manner, IL6 is considered as a main pro-inflammatory cytokine during the acute phase of neuroinflammation. Several research groups described a transient IL6 level increase after the peripheral nerve injury. Cao and coll. (Cao et al., 2009) observed an elevation of IL6 protein expression with a peak at the day 1 after L5 nerve transection, which was maintained at the day 3, but no significant difference was detected between the ipsilateral and the contralateral spinal cord on the day 7. Regarding the chronology of events and the time-point used for western blot analyses, IL6 may exhibit an earlier peak of expression, before its levels decreased to reach similar to “non-neuropathic” conditions, corresponding in our model to the contralateral side levels.

Our western blot experiments indicated also that losartan treatment inhibited SNL-induced AT1R overexpression in the ipsilateral spinal cord. Those results suggested

that AT1R activation might be also involved in the inflammatory mechanisms at the spinal cord level. It is important to mention that recently, a study based on the comparison of specificity of six commercial anti-AT1R antibodies from different suppliers, highlighted the lack of specificity of all anti-AT1R antibodies tested through western blot and immunohistochemistry tests in wild type and AT1R knocked-out mice (Benicky et al., 2012). Further experiments regarding the gene expression may allow us to confirm our results obtained from western blot analyses.

Peripheral nerve injury is known to lead to neuroimmune response both in central and peripheral nervous systems, including activation of resident immune-like cells (microglia in spinal cord or satellite glial cells in DRG) and /or invasion of immunocompetent cells of haematogenous origin (invaded macrophages). The peak of neuroimmune response may vary in different models of peripheral nerve injury (Ton et al., 2013), but in the SNL model, it is commonly observed approximately in a week after the surgery (Churi et al., 2008, Austin and Moalem-Taylor, 2010). In our study, tight ligation of L5 spinal nerve promoted the infiltration of blood-derived macrophages into ipsilateral L5 DRG and gDR on the day 7 after the SNL, as well as microglia activation in the ipsilateral spinal cord, reflected in significant increase of OX42 protein levels.

According to our immunohistochemistry results, systemic losartan treatment significantly attenuated CD68-positive macrophage accumulation in ipsilateral DRG and gDR. Several research groups based their experiments of effective anti-inflammatory treatments for different models by using AT1R blockade strategies. For example, AT1R blockers were tested with encouraging results in such pathophysiological conditions as dementia (Wang et al., 2007), atherosclerosis (Yamamoto et al., 2015), or diabetes (Nakamura et al., 2009), and other diseases with the inflammatory origin. Surprisingly, the different blockers may exhibit anti-inflammatory activity via distinct pathways. In fact, losartan acts as a proantagonist in the AT1R blockade since it doesn't bind the Ang II receptor itself. After the administration, losartan is catabolized into two metabolites: EXP3174 and EXP3179. EXP3174 has been described as the proper antagonist molecule, acting as a competitive antagonist (Lo et al., 1995, Stearns et al., 1995), while EXP3179 doesn't interfere with any AngII binding sites (Kramer et al., 2002). In the present study, we should not exclude the possibility EXP3179 metabolite may activate different transduction pathways, independently to AT1R antagonism. Marshall and coll. studied the structure-function relationship of several sartans and their binding affinity to the nuclear receptor for vitamin D and PPARγ, and revealed that some sartans including losartan exhibited a significant affinity to these receptors (Kramer et al., 2002). Moreover, other

studies demonstrated the capacity of losartan to activate PPARγ transduction pathways (An et al., 2010, Hamano et al., 2011, Matsumura et al., 2011). We hypothesize, that in our study, the effect of losartan treatment on CD68-positive macrophages might be mediated by similar mechanism, as its metabolite EXP3179 binds to PPARγ in invaded macrophages (Gordon, 2003). Recently, PPARγ have been intensively studied for their role in macrophage polarization. A growing number of studies confirm, that PPARγ activation triggers macrophage polarity shift from M1 (pro-inflammatory) to M2 (anti-inflammatory) phenotype, thus reducing local neuroinflammation and neuropathic pain behavior (Churi et al., 2008, Hasegawa-Moriyama et al., 2012).

In a wider range of new neuropathic pain drug development, the emerging data based on strategies involving the RAS, especially via the blockade of the angiotensin II signaling, seem to be very promising. Recently, two research groups reported the analgesic effects of small molecule AT2R antagonists in a peripheral nerve injury and anti-retroviral neuropathic pain models in rats (Smith et al., 2013, Smith et al., 2014), as well in postherpetic neuralgia patients (Rice et al., 2014). In particular, the advanced study of Rice and coll. by a randomized, double-blind, placebo-controlled phase II clinical trial, showed a superior relief of postherpetic neuralgia with no serious side effects in patients treated with EMA401, a small molecule AT2R antagonist, when compared with patients who received the placebo at the end of 28 days of treatment (Rice et al., 2014).

Taken together and considering a larger context, losartan treatment might be effective against neuroinflammation-driven pathologies. Hence, it represents a promising strategy for the anti-inflammatory treatments development.

6.2 The effect of losartan treatment on neuroinflammatory changes in the model of chemotherapy-induced peripheral neuropathy

This original study was designed to describe the effect of systemic LOS treatment on the development and maintenance of chemotherapy-induced peripheral neuropathy and to reveal the possible mechanisms underlying LOS anti-inflammatory action. The

outcome of the LOS treatment is assessed on the behavioral and molecular levels; it is described in the manuscript (Publication B) and covers the aim 2 of this Thesis.

Systemic treatment with paclitaxel leads to the development of peripheral neuropathy in rats, which is characterized by mechanical allodynia occurring within days and lasting up to few weeks after the start of the paclitaxel treatment (Polomano et al., 2001). In our study, we used this animal model to evaluate the effect of losartan treatment on the development and maintenance of the PIPN. Behavioral measurements confirmed the presence of the PIPN in the PAC group, as well as its abolishment in the LOS group at most time points, indicating positive anti-neuropathic effect of losartan treatment.

When applied systemically, paclitaxel cross the blood-brain barrier poorly, but penetrates the blood-nerve barrier and accumulates predominantly in DRGs, where persists in high concentrations up to 10 days and more after the last paclitaxel injection (Xiao et al., 2011). For our study, we consider 10 days of paclitaxel treatment as acute developmental phase of the PIPN, when paclitaxel was gradually accumulating in DRG, reaching its maximal concentration after the last injection (day 9) and affecting predominantly DRG neurons and SGC. We also assume that during the next phase, referred in this study as chronic, the maintenance of the PIPN was mediated not only by neuroinflammation in DRGs but also by pathological changes on the spinal cord level, especially by neuroimmune modulation of synaptic transmission in the spinal cord dorsal horn. According to behavioral tests, mechanical allodynia was developing during acute phase and reached its maximum in chronic phase in the PAC group, while in the LOS group the behavioral changes seem to be two-phasic. During the acute phase of the PIPN, the PWL to mechanical stimuli tend to decrease, with the exception of early measurement on the day 7, when PWT did not differ from the CTRL values, but were significantly higher than PAC group values. This discrepancy may be explained by low ability of losartan treatment to prevent from acute changes induced by paclitaxel directly in the DRGs. On the day 7, when we observed analgesic effect of losartan treatment on behavioral signs of the PIPN, the experimental animals received only 3 injections of paclitaxel, hence the concentration of the drug was relatively low in the DRGs. However, after the drug delivery was accomplished and the concentration of paclitaxel in the DRGs reached its maximum, the protective effect of losartan treatment was not enough to prevent the acute changes in PWT. In spite of uncertain effect of losartan treatment over acute phase, we observed significant changes in the LOS group during the chronic phase, when compared to the PAC group. These data indicate preventive analgesic effect of losartan treatment mainly during chronic phase of the PIPN, which is hypothesized to be driven not by paclitaxel itself, but

by secondary pathological neuroinflammatory changes in peripheral and central levels of nervous system.

Regarding the acute phase of the PIPN, values of inflammatory proteins expression and specific mRNA levels, measured on the day 10, indicate the neuroinflammatory changes predominantly in DRGs. Our results show a significant elevation of GFAP protein levels as well as key inflammatory chemokine CCL2, produced by activated SGC in DRGs of PAC rats. As it is well known, CCL2 is the main chemoattractant agent, which attracts blood-derived immune cells of monocyte line (Leonard et al., 1991). Most anti-PIPN strategies are currently focused on neuronal mechanisms, while growing number of studies indicate the essential role of macrophage infiltration in the PIPN development. It is necessary to mention that macrophage activation/infiltration profile is highly dependent on the chosen drug, administration type, dosage and time schedule. For paclitaxel, macrophage activation in DRG may occur already on the day 3 after the start of the treatment (for 4x2 mg/kg, *i.p.* (Zhang et al., 2016) or 3x8 mg/kg i.p. dosage (Liu et al., 2010)) or during the second week (for 2x16 mg/kg i.p. (Nishida et al., 2008) and 18 mg/kg i.v. (Peters et al., 2007)). Once in the tissues, monocyte-derived macrophages respond to microenvironmental cues that determine whether they contribute to the establishment of local inflammatory response (M1-like macrophages) or to it resolution (M2-like subtype). In our study, we observed the presence of activated macrophages in DRGs of the PAC group already on the Day 10, reflected in elevated protein levels of CD11b and CD68 along with mRNA levels for CD68 when compared to the VEH group. The presence of activated macrophages was also confirmed by increased protein and mRNA levels of TNFα, the main cytokine product of M1 macrophages (Martinez and Gordon, 2014), as well as its specific receptor TNFR1protein, which is known to be expressed on DRG neurons and SGCs during inflammatory process, thus making those cells more susceptible to TNFα and enhancing the development of neuroinflammation. Losartan treatment abolished paclitaxel-induced overexpression of GFAP, CCL2, CD68, TNFα and TNFR1 proteins, as well as mRNA for CD68 and TNFα in DRG. On the contrary, CD11b protein levels were significantly increased in the LOS group in the same manner as in the PAC group. While CD68, also called ED1, is a lysosomal protein expressed in phagocyting M1 macrophages (Dijkstra et al., 1985), CD11b, also known as OX42, is an integrin alpha M membrane protein, expressed by innate immune system cells including microglia, "resident" and "inflammatory" monocytes (Gordon and Taylor, 2005). Therefore, CD11b may be used as a marker of both M1 and M2 macrophages. As the levels of CD68 in the LOS group were significantly lowered, we may hypothesize, that at the time point, the main effect of the LOS treatment was in

suppression of phagocyting activity of macrophages, but not in the prevention of macrophage migration to the DRGs. We also measured a significant paclitaxel-induced microglia activation (CD11b protein), and iNOS mRNA decrease in the SCDH, which both were abolished in the LOS group. Paclitaxel-induced elevation in CD11b levels indicate a microgliosis in the SCDH. It is known that activated microglia produces pro-inflammatory mediators, including NO, and high concentrations of NO reciprocally reduce iNOS mRNA expression in microglia (Sheffler et al., 1995, Li et al., 2005). Our data suggest that anti-inflammatory effect of losartan applies not only on activated macrophages in DRG, but also on microglia in CNS (directly or secondary by attenuation of pro-inflammatory signal input from DRG).

Losartan is a widely used antihypertensive drug, which blocks AT1R. However, macrophages do not express angiotensin receptors at their surface. It is important to emphasize, that AT1R are present in endothelial cells and their role in BBB integrity has been widely discussed recently. In particular, activation of AT1R is known to lead to the enhancement of BBB permeability and application of AT1R antagonists was able to effectively prevent BBB disruption and suppress inflammatory events in the CNS structures in different models (Saavedra, 2012, Biancardi et al., 2014). However, the main effect of losartan treatment, observed in our experiments, was on the attenuation of phagocytic activity in already invaded macrophages. Different properties of losartan metabolites EXP3174 and EXP3179 may explain the effect on macrophages. Losartan acts as a proantagonist in the AT1R blockade since it does not bind the AT1R itself. After the administration, losartan is catabolized into two metabolites: EXP3174 and EXP3179. EXP3174 has been described as the proper antagonist molecule, acting as a competitive antagonist (Lo et al., 1995, Stearns et al., 1995), while EXP3179 does not interfere with any Angiotensin II binding sites (Kramer et al., 2002), but mediates the activation of PPARγ receptors (Rossi, 2009). Since PPARγ response elements were identified in the promoter region of *Arg1* gene, one of the suggested mechanisms of PPARγ agonist-induced macrophage polarization shift is direct upregulation of Arginase 1 along with downregulation of TNFα and NO expression by macrophages (Song et al., 2016). We assume that in our study the anti-inflammatory effect of losartan treatment was mediated mainly by its EXP3179 metabolite and PPARγ agonism.

The proofs of our hypothesis were more evident during the chronic phase of the PIPN. In DRGs of paclitaxel-treated animals, we observed the presence of macrophages reflected in overexpression of CD11b protein on the day 21 after the first paclitaxel injection. Increased levels of chemoattractant CCL2 protein along with CD68, and

macrophage pro-inflammatory product IL6, were significantly attenuated in the LOS group, indicating anti-inflammatory effect of losartan treatment. Regarding mRNA levels in DRG, it is clear that paclitaxel treatment induced an upregulation of markers indicating M1-like macrophage presence (CCR2, CD68, CD11b, NOX2, iNOS, CD64), which was abolished with losartan treatment. Our data suggest that paclitaxel treatment induce the chemotactic invasion of CCR2+ monocyte subset (confirmed by the increase of CD11b mRNA) towards CCL2 chemokine in DRGs. These cells exhibited active phagocytosis and production of ROS and NO that is reflected in the elevation of CD68, NOX2, and iNOS mRNA expressions respectively. CD64, a Fc receptor for immunoglobulin G, is expressed not only in macrophages, but also it is upregulated in nociceptive DRG neurons during BBB dysfunction (Andoh and Kuraishi, 2004b, a). Thus, the increase in CD64 mRNA expression in PAC group may reflect both M1 macrophage presence and DRG neuronal activation during chronic phase of the PIPN.

On the other side, losartan treatment resulted in robust increase in expression of M2-like macrophage markers ARG1 and IL10, confirming the effect of losartan on macrophage polarization state. IL10 is an anti-inflammatory cytokine produced by M2 macrophages. Its pro-resolving properties include also macrophage deactivation, characterized by inhibition of pro-inflammatory cytokines production via destabilization of mRNA transcripts for TNF or IL1β (Vanderwall et al., 2018), increased anti-inflammatory cytokine production and reduced major histocompatibility complex class II expression (Gordon and Taylor, 2005). Elevated protein levels of IL10 in spinal cord are known to produce pain relief in neuropathic pain models including PIPN (Ledeboer et al., 2005, Vanderwall et al., 2018). Moreover, PIPN-induced mechanical allodynia may be prevented and/or reversed by the intrathecal IL10 therapy in rats (Ledeboer et al., 2007). The elevated levels of IL10 mRNA in DRGs of the LOS rats correlate well with the abolishment of allodynic state in PAC-treated rats in our experiments.

It is important to mention that the expression of Arginase 1, which is widely viewed as a typical marker of M2 macrophages (Gordon, 2003, Martinez and Gordon, 2014), is also regulated by PPARγ (Odegaard et al., 2007). Several studies confirmed that activation of PPARγ, e.g. with synthetic agonists, leads to the substantial elevation of Arginase 1 and IL10 protein and mRNA levels in macrophages (Bouhlel et al., 2007, Gao et al., 2015, Su et al., 2017). Additionally, the elevation of PPARγ mRNA levels in the LOS group most probably indicates a positive feedback loop mechanism (Wakabayashi et al., 2009). These data further confirm the hypothesis that anti-inflammatory effect of losartan in our study is mainly mediated through PPARγ receptors.

Anti-inflammatory effect of losartan treatment on the DRG was also reflected in the attenuation of paclitaxel-induced inflammatory response in the SCDH during chronic phase of the PIPN. Losartan treatment prevented paclitaxel-induced astrogliosis, as well as the expression of main pro-nociceptive and pro-inflammatory glial mediators – CCL2 and TNFα. It is known, that CCL2 and TNFα play important role in central sensitization and pathological pain development through the modulation of synaptic transmission in the SCDH (Spicarova et al., 2011, Spicarova et al., 2014a). The decrease in CCL2 and TNFα levels resulted in significant attenuation in behavioral responses to mechanical stimulations, when compared with the PAC group.

Our study demonstrates significant anti-inflammatory and pro-resolving effects of losartan treatment in the model of the PIPN. Considering the toxicity of PPARγ complete agonists, the safety profile of losartan partial PPARγ agonism with mainly positive outcomes may be used as a novel treatment strategy for PIPN patients.

6.3 The involvement of spinal TRPV1 receptors in neuronal activation induced by chemotherapy

In this study, we aimed to investigate the role of spinal TRPV1 receptors in the mechanism of PAC-induced neuronal activation. We used immunohistochemical approach to detect the PAC-induced expression of c-Fos protein in the nuclei of spinal dorsal horn neurons with or without TRPV1 receptor inhibitors. It covers the aim 3 (Publication C).

A growing number of studies focused on the induction of c-Fos expression in neuron nuclei confirm a wide diversity of peripheral stimuli that may trigger the upregulation of the immediate early gene *c-fos* and its protein product c-Fos in dorsal horn neurons. These stimuli include mechanical, thermal, chemical and electrical stimulations (Coggeshall, 2005). The administration of paclitaxel is known to induce neurotoxic and inflammatory changes in neurons, leading to the PIPN (Makker et al., 2017). Since c-Fos is considered as a signal transducer linking extracellular events and intracellular signaling cascades, we decided to test the effect paclitaxel on c-Fos expression in neuron nuclei in superficial laminae of dorsal horn, a nociceptive area of spinal cord. As it was mentioned above, c-Fos is a very unstable protein with rapid nuclear turnover (Vesely et al., 2009).

The expression of *c-fos* mRNA can be rapidly activated within a few minutes after the stimulation, subsequently translated into c-Fos protein, which concentration peaks at 1-2 hours after stimulus application (Gao and Ji, 2009). It is important to emphasize that we measured c-Fos immunoreactivity 1 hour after the incubation with paclitaxel. In this work, we demonstrate for the first time that paclitaxel in relatively low concentration activates the expression of c-Fos in the superficial dorsal horn neurons nuclei, indicating one of the possible mechanisms of PIPN development. It was shown recently that systemic PAC treatment leads to effective concentrations of PAC in the nervous system (Yan et al., 2015). The concentration of PAC used for our experiments is thus adequate to that, which may be reached in the spinal cord after systemic PAC administration. The concentration of SB366791 (10μM) was set according to previous published data (Ferrini et al., 2007, Spicarova and Palecek, 2009).

Due to the specificity of our *in vitro* study design, only central branches of primary afferents were present in our spinal cord slices. Thus, the effect of PAC on spinal dorsal horn neurons had to be mediated either by the activation of presynaptic endings and/or by the direct effect on the postsynaptic neurons. Recently, it was shown that systemic administration of paclitaxel results in thermal hypersensitivity and increased expression of TRPV1 receptors in DRG neurons (Hara et al., 2013). It was also documented (Li et al., 2015) that i) intrathecal application of TRPV1 antagonist attenuate PAC-induced hypersensitivity; ii) TRPV1 and TLR4 receptors are co-expressed in DRG neurons; and iii) PAC treatment-induced increase of TRPV1 expressing DRG neurons is dependent on activation of the TLR4. Moreover, PAC-induced increase in the responsiveness of TRPV1 receptors to capsaicin is also dependent on the TLR4 signaling pathway (Li et al., 2014, Li et al., 2015). These findings suggest that in our study, the effect of PAC on the neuronal c-Fos expression was mediated by the activation of presynaptic TLR4 and TRPV1 receptors.

Several studies demonstrate that the activation of TLR4 receptors may trigger *c-fos* gene activation directly (Introna et al., 1986, Guha and Mackman, 2001). However, at the spinal cord level, TLR4 receptors are expressed predominantly by glial cells (Saito et al., 2010, Li et al., 2014), but not by neurons (Li et al., 2014). The activation of microglia leads to the release of cytokines and chemokines (Saito et al., 2010), which in turn may potentiate presynaptic TRPV1 receptor function (Spicarova et al., 2011, Spicarova et al., 2014a). The effect of PAC treatment in this study was thus most likely mediated through presynaptic and/or glial TLR4 receptors, which induced the activation of presynaptic TRPV1 receptors. This signaling cascade led to the increased release of neurotransmitters and neuromodulators such as glutamate, substance P and CGRP, followed by activation of

postsynaptic neurons and c-Fos expression. This hypothesis is in agreement with electrophysiological data, measured under similar *in vitro* conditions, when increased spontaneous and miniature excitatory postsynaptic currents frequency after PAC treatment were dependent on TRPV1 receptors activation (Li et al., 2015).

In our experiments, both antagonists significantly diminished the PAC-induced c-Fos expression. However, application of SB366791 alone, but not AMG9810, induced a modest but still significant increase in c-Fos expression, when compared to the control group. We assume it is possible that this discrepancy between the two TRPV1 receptor antagonists will be due to different functional properties of these antagonists. AMG9810 belongs to a so called "group A TRPV1 antagonists", which are effective against both capsaicin and proton activation of TRPV1 receptor. On the contrary, SB366791 is a "group B" antagonist, which blocks capsaicin, but not proton, activation of TRPV1 receptor (Gavva et al., 2005). Moreover, at higher concentrations (>0.3 μM), SB366791 potentiates pH5-induced activation of TRPV1 (Gavva et al., 2005). It is necessary to point out that in our study a concentration of 10μM was used; therefore, it is possible that higher concentration of protons, originated from damaged tissue during spinal cord slices preparation, will be reflected in potentiated activation of TRPV1 receptors, when slices are incubated with SB366971.

Taking together, these findings further confirm the important role of spinal cord TRPV1 receptors in the development of pathological pain states, including neuropathic pain after paclitaxel treatment.

6.4 The role of spinal TRPV1 receptors in CCL2-induced pain hypersensitivity and spinal neuronal activation in rats

This study describes the pronociceptive effect of intrathecal CCL2 application in rats and CCL2-induced neuronal activation in spinal cord and investigates the role of spinal TRPV1 receptors in these processes. It covers the aim 4 (Publication D).

CCL2 is considered as one of the main pronociceptive markers after the peripheral nerve injury. The increased CCL2 expression by DRG neurons was described in different neuropathic pain models, including nerve constriction (Zhang and De Koninck, 2006), CCI and SNL (Jeon et al., 2009). Co-localization of CCL2 and the substance P, CGRP and TRPV1

in DRG neurons suggests the CCL2 synthesis mainly by nociceptive neurons (Dansereau et al., 2008). After the peripheral nerve injury, CCL2 is synthesized in the affected DRG neurons, transported via central terminals, and acts as pronociceptive neuromodulator in the SCDH (Van Steenwinckel et al., 2011). In our experiments, we used intrathecal CCL2 application that induced pronounced thermal hyperalgesia and mechanical allodynia, which are in the agreement of previously published observations (Tanaka et al., 2004, Dansereau et al., 2008, Gao et al., 2009, Thacker et al., 2009). Several studies demonstrate a long-term CCL2-induced mechanical hypersensitivity (Thacker et al., 2009); we observed changes in mechanical sensitivity only during several hours after the application. We hypothesize, that the discrepancies may be explained by the different experiment set up, rat strains and/or dosage used. Regarding thermal hyperalgesia, Thacker and colleagues (Thacker et al., 2009) did not observe CCL2-induced thermal hypersensitivity, when measured 1 day after the intraspinal application. In our study, we measured transient thermal hyperalgesia that was the most pronounced several hours after the CCL2 injection and returned to the basal values 24 hours after. Our observations are similar to those in other studies (Gao et al., 2009, Thacker et al., 2009).

Pretreatment with TRPV1 antagonist SB366791 significantly abolished the development of CCL2-induced thermal hyperalgesia, while the mechanical allodynia was not affected. These observations suggest that spinal TRPV1 receptors play an important role in the development of CCL2-induced thermal hyperalgesia, but they are not involved in the development of hypersensitivity to mechanical stimuli after CCL2 *i.t.* application. Our results are in accordance with previously published findings. Cavanaugh and colleagues (Cavanaugh et al., 2009) demonstrate that pharmacological ablation of the central terminals of TRPV1-expressing peptidergic nociceptors results in substantial loss of noxious heat, but not mechanical pain sensitivity. On the other side, they observed a selective reduction of mechanical sensitivity after genetic ablation of unmyelinated sensory neurons expressing the G protein-coupled receptor Mrgprd, which consist approximately 90% of the cutaneous nonpeptidergic nociceptors (Cavanaugh et al., 2009). These results further support the hypothesis that spinal TRPV1 receptors are involved primarily in the development of noxious heat hypersensitivity, but not of mechanical allodynia.

In our study, CCL2 was applied intrathecally, and pronociceptive effect was present for several hours after the application, reflected in decreased PWL and PWT. Pain hypersensitivity, induced by the intrathecal application of CCL2, is most likely mediated by spinal CCR2 receptors (Cavanaugh et al., 2009). In this case, CCR2 receptors expressed by

SCDH neurons (Gosselin et al., 2005, Gao et al., 2009), astrocytes (Knerlich-Lukoschus et al., 2008) and microglia (Siebert et al., 2000) represent the possible target for administered CCL2. In support of this hypothesis, an *i.t.* injection of CCL2 activated spinal microglia in wild type, but not in CCR2 knockout mice (Zhang et al., 2007, Thacker et al., 2009). CCL2 application results in the activation of spinal microglia within hours (Zhang et al., 2007, Thacker et al., 2009) and in rapid (<5min) activation of pERK in superficial SCDH neurons (Gao et al., 2009), which was confirmed also with our immunohistochemical experiments described in the next chapter. Astrocytes may be also involved in the CCL2/CCR2 signalization, as CCR2-mediated ERK phosphorylation in spinal astrocytes leads to the synthesis and release of pro-nociceptive mediators including IL-1β, and to the subsequent release of CCL2 (Old and Malcangio, 2012). Besides the direct cell activation by CCL2/CCR2 signaling, CCL2 may enhance the inward currents elicited by both AMPA and NMDA (Gao et al., 2009) and inhibit GABA currents (Gosselin et al., 2005).

Regarding the exact mechanism of CCL2 action on the spinal TRPV1 receptors, we believe that it is mediated also by CCR2 receptors expressed on the presynaptic endings in the SCDH. Upon activation, CCR2 receptor mediates its functions through the activation of phospholipase C (PLC) and further hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2). As TRPV1 receptors are tonicaly blocked by PIP2, its hydrolysis enables TRPV1 receptors to become active (Chuang et al., 2001). Additionally, TRPV1 sensitization/activation state may be also modulated via phosphorylation by protein kinase C (PKC), which increases the channel open probability for TRPV1 receptors (Huang et al., 2006, Jung et al., 2008).

The activation of ERK1/ERK2, reflected in its phosphorylation (pERK), is also an often-used marker of cell activation after noxious peripheral stimulation (Ji et al., 1999). The chemokine CCL2 plays a pivotal role in the development of neuropathic pain, especially after peripheral injury (Gao et al., 2009, Jeon et al., 2009, Fu et al., 2010, Van Steenwinckel et al., 2011). While the involvement of CCL2/CCR2 signaling was described well in the periphery, the effect of spinal CCL2 on the development of central sensitization is poorly understood. At the spinal cord level, the CCL2 may be released from the primary afferents (Jung et al., 2008, Van Steenwinckel et al., 2011) and/or by spinal astrocytes (Gao et al., 2009). CCR2 are expressed by small and medium-sized DRG neurons (White et al., 2005, Jung et al., 2008), dorsal horn neurons (Gao et al., 2009), astrocytes (Knerlich-Lukoschus et al., 2008) and microglia (Siebert et al., 2000, Old and Malcangio, 2012).

In our study, the incubation of spinal cord slices in CCL2-containing medium induced a significant increase in the pERK immunoreactivity in the SCDH, which is in line with previous demonstrations, where the same concentration of CCL2 was used (Gao et al., 2009). The electrophysiological results in this study (Publication D) indicate that CCL2-induced effect in the SCDH is mediated via G-protein coupled CCR2 receptors on the presynaptic endings, the activation of which was followed by TRPV1-dependent glutamate release from the synaptic terminals. Indeed, the preincubation with TRPV1 receptor antagonist SB366791 significantly abolished the CCL2-induced pERK activation in the SCDH. Moreover, the preventive effect of SB366791 corresponds also with its anti-hyperalgesic effect in the behavioral experiments with intrathecal administration of CCL2 and SB366791/CCL2 combination described in previous chapter.

Taking together mentioned direct or indirect effects of CCL2, it is clear that CCL2/CCR2 signaling plays an important role in mechanisms of central sensitization, resulting in the development of pathological pain states. Preventive effect of TRPV1 receptor antagonist on CCL2-induced thermal hyperalgesia, but not mechanical allodynia, suggested distinct underlying mechanisms of these two behavioral outcomes. Our data suggest also that spinal TRPV1 receptors may play an important role in CCL2-induced neuronal activation, which may lead to the development of pathological pain states.

7. CONCLUSIONS

Following conclusions were made according to the specific aims of the Thesis:

1) Systemic losartan treatment exerts protective effects against the neuroinflammatory and neuropathic changes after the peripheral nerve injury. However, losartan is ineffective against the SNL-induced mechanical allodynia, suggesting distinct mechanism of its development, when compared to the thermal hyperalgesia. Nevertheless, losartan treatment represents a promising strategy for the anti-inflammatory treatments development.

2) Systemic losartan treatment is also effective against chemotherapy-induced neuropathic changes. Considering our findings and a wider context, anti-neuropathic effect of losartan is the most likely mediated by the PPARγ receptors. Along with analgesic and anti-inflammatory effects of losartan, we demonstrated also a significant pro-resolving effect, which may be of particular interest as a novel treatment strategy for PIPN patients.

3) Our results have shown an important role of TRPV1 receptors in increased activation of dorsal horn neurons (demonstrated by increased c-Fos expression) after application of paclitaxel. This further confirms the role of TRPV1 receptors in the development of neuropathic pain following Paclitaxel treatment.

4) Our study demonstrates a significant role of spinal CCL2/CCR2 signaling in the development of acute pain hypersensitivity. Here, we also demonstrate that spinal TRPV1 receptors play a substantial role in the development of CCL2-induced acute thermal hyperalgesia, revealing a new possible target for anti-hyperalgesic treatment development. Spinal TRPV1 receptors are also involved in the mechanisms of CCL2-induced neuronal activation at the level of the SCDH. Hence, it is plausible to assume the active role of spinal TRPV1 receptors in the central sensitization phenomenon, which may further result in the development of neuropathic pain syndrome.

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LIST OF ALL PUBLICATIONS

Publication A. **Kalynovska N**, Diallo M, Palecek J. *Losartan treatment attenuates the development of neuropathic thermal hyperalgesia induced by peripheral nerve injury in rats*. Life Sci. 2019 Mar 1; 220:147-155. **IF = 3.2**

My personal contribution to this paper includes supervision over the overall experiments, performing of all behavioral experiments, sample preparations, immunohistochemistry experiments and participation in the western blot experiments. I also performed final data evaluation, statistical analysis, creation of figures, and writing a manuscript.

Publication B. ***Submitted manuscript.*** **Kalynovska N**, Diallo M, Sotakova-Kasparova D, Palecek J. *Losartan treatment exerts anti-neuropathic and anti-inflammatory effects in the model of paclitaxel-induced peripheral neuropathy in rats*.

My personal contribution to this prepared paper includes supervision over the overall experiments, performing of behavioral experiments, sample preparations, ELISA, the majority of RT PCR, and participation in western blot experiments. I also performed complex analysis of all the data and its interpretation, created the graphs, and wrote the manuscript.

Publication C. **Kalynovska N**, Adamek P, Palecek J. *TRPV1 receptors contribute to mediate paclitaxel-induced c-Fos expression in spinal cord dorsal horn neurons.* Physiol Res. 2017 Jul 18; 66(3):549-552. **IF = 1.3**

My personal contribution to this article consists of acute spinal cord slice preparation and incubation, immunohistochemistry, data evaluation and interpretation, creation of graphs, and writing of manuscript.

Publication D. Spicarova D, Adamek P, **Kalynovska N**, Mrozkova P, Palecek J. *TRPV1 receptor inhibition decreases CCL2-induced hyperalgesia.* Neuropharmacology. 2014 Jun; 81:75-84. **IF = 4.3**

My personal contribution to this article includes the performing of all behavioral experiments, including the implantation of lumbosacral catheters and intrathecal injections, behavioral testing, and data interpretation. I also participated in the immunohistochemical experiments, data evaluation, and manuscript preparation.

APPENDIX

Publications enclosed in full (Publication A, Publication C, Publication D)