Charles University

Faculty of Science

Study programme: Animal physiology

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THE ROLE OF NOCICEPTIVE SYNAPTIC TRANSMISSION MODULATION AT THE SPINAL CORD LEVEL IN DIFFERENT PAIN STATES

VÝZNAM MODULACE NOCICEPTIVNÍHO SYNAPTICKÉHO PŘENOSU NA MÍŠNÍ ÚROVNI ZA RŮZNÝCH BOLESTIVÝCH STAVŮ

DOCTORAL THESIS

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Praha 2019

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Acknowledgement

Abstrakt

Bolest je běžným příznakem mnoha klinických syndromů a nemocí. Zejména léčba bolestí neuropatického původu představuje závažný medicínský problém, neboť dostupná analgesie je v řadě případů neúčinná, nebo má výrazné nežádoucí účinky. Vývoj nových analgetických postupů a úspěšná léčba bolesti proto vyžaduje podrobnou znalost mechanizmů vzniku akutních i chronických bolestivých stavů. Proces vzniku, kódování a přenosu signálů o bolestivých podnětech zprostředkovává nociceptivní systém, který je klíčový pro vznik vjemu bolesti v mozku. Modulace nociceptivního synaptického přenosu v zadním rohu míšním představuje důležitý mechanismus ve vývoji a udržování různých patologických stavů bolesti.

Tato disertační práce se zaměřila na zkoumání a objasnění některých mechanismů podílejících se na zpracování a modulaci míšního nociceptivního synaptického přenosu u různých modelů bolestivých stavů. Hlavní pozornost byla věnována studiu následujících otázek: (I.) Jakou úlohu mají TRPV1 (Transient Receptor Potential Vanilloid type 1) kanály, TLR4 (Toll-Like Receptor 4) a PI3K (fosfatidylinositol 3-kináza) v rozvoji neuropatické bolesti po podání chemoterapeutika paclitaxel (PAC) v akutním *in vitro* a subchronickém *in vivo* myším modelu PAC-indukované periferní neuropatie (PIPN)? (II.) Do jaké míry je ovlivněna míšní inhibiční synaptická kontrola u animálního modelu PIPN, akutního periferního zánětu a chronického konstrikčního poranění sedacího nervu (CCI) u transgenního kmene myši VGAT-ChR2-eYFP? (III.) Jak ovlivní inhibice sodíkového kanálu $Na_v1.7$ specifickým antagonistou protoxinem II spinální nociceptivní signalizaci u modelu termálního poškození kůže? (IV.) Jak *N*-arachidonoylfosfatidylethanolamin (20:4-NAPE), prekurzor anandamidu (AEA), moduluje nociceptivní synaptický přenos u modelu akutního periferního zánětu a jakou roli hraje kanabinoidní receptor 1 (CB_1) v tomto procesu?

Pro zkoumání těchto cílů byla využívána metoda patch-clamp v konfiguraci snímání z celé buňky, která nám umožnila snímání excitačních či inhibičních postsynaptických proudů (EPSC, resp. IPSC). Dále bylo využíváno behaviorální měření mechanické či tepelné citlivosti a imunohistochemická analýza.

Naše výsledky ukázaly, že: (I.) Přímá funkční interakce mezi TLR4 a TRPV1 receptory, zejména prostřednictvím PI3K signalizace, hraje důležitou roli v (*a*) PAC-indukovaném nárůstu frekvence miniaturních EPSC v neuronech zadního rohu míšního, (*b*) v modulaci a tachyfylaxi kapsaicinem vyvolaných odpovědí zprostředkovaných TRPV1 kanály na presynaptických zakončeních primárních aferentů v míše a (*c*) v PAC-indukované mechanické alodynii. Všechny tyto PAC-indukované změny bylo možné zablokovat inhibitorem PI3K wortmanninem. Mechanismus závislý na TRPV1 je také nezbytný pro PAC-indukované zvýšení exprese proteinu c-Fos v neuronech zadního rohu. (II.) Naše předběžné výsledky poukazují na významnou roli disinhibice v zadním rohu míšním v rozvoji mechanické allodynie ve všech testovaných modelech bolestivých stavů (PIPN, periferní zánět a CCI). (III.) Inhibitor $Na_v1.7$ kanálu protoxin II významně omezil zvýšenou excitační aktivitu v populaci nociceptivních, kapsaicin-senzitivních neuronů u modelu

termálního poškození kůže. (IV.) Potvrdili jsme hypotézu, že 20:4-NAPE slouží jako zdroj pro endogenní syntézu AEA v míše *in vitro* a že inhibiční účinek 20:4-NAPE je zprostředkován mechanismem závislým na CB_1. Tento analgetický účinek 20:4-NAPE zprostředkovaný CB_1 receptory je však za zánětlivých stavů částečně modifikován dalším mechanismem závislým na aktivaci TRPV1 kanálů.

V souhrnu naše data podporují názor, že za různých patologických bolestivých stavů dochází k výrazné modulaci nociceptivního synaptického přenosu na míšní úrovni. Ukázali jsme také, že vhodná intervence a farmakologická léčba mohou pomoci zmírnit zvýšený nociceptivní přenos, nebo utlumit behaviorální projevy související s bolestí u zvířat.

Porozumění mechanismům modulace nociceptivního synaptického přenosu je nezbytným předpokladem pro zlepšení terapeutických přístupů pro léčbu bolestivých stavů v budoucnosti.

Klíčová slova:

Bolest, nocicepce, synaptický přenos, neuropatická bolest, paclitaxel, TRPV1, kapsaicin, TLR4, PI3K, $Na_v1.7$, CB_1, anandamid.

Abstract

Pain is a common symptom of many clinical syndromes and diseases. In particular, the treatment of neuropathic pain represents a serious public health issue because currently available analgesia is ineffective in many cases or it has adverse effects. Treatment of pain-related suffering requires knowledge of how pain signals are initially generated and subsequently transmitted by the nervous system. A nociceptive system plays a key role in this process of encoding and transmission of pain signals. Modulation of the nociceptive synaptic transmission in the spinal cord dorsal horn represents an important mechanism in the development and maintenance of different pathological pain states.

This doctoral thesis has aimed to investigate and clarify some of the mechanisms involved in the modulation of the spinal nociceptive processing in different pain states. The main attention was paid to study the following issues: (I.) Which is the role of Transient Receptor Potential Vanilloid type 1 channels (TRPV1), Toll-Like Receptors 4 (TLR4), and phosphatidylinositol 3-kinase (PI3K) in the development of neuropathic pain induced by paclitaxel (PAC) chemotherapy in acute *in vitro*, and subchronic *in vivo* murine model of PAC-induced peripheral neuropathy (PIPN)? (II.) How is affected spinal inhibitory synaptic control under different pain states, using VGAT-ChR2-eYFP transgenic mice model of PIPN, acute peripheral inflammation, and chronic constriction injury injury (CCI) of the sciatic nerve? (III.) How does the $Na_v1.7$ receptor blocker protoxin II affect the spinal nociceptive signaling in the model of burn injury? (IV.) How 20:4-NAPE (*N*-arachidonoylphosphatidylethanolamine), the precursor of anandamide (AEA), modulates the nociceptive synaptic transmission under the acute inflammatory condition and which role plays cannabinoid receptor 1 (CB_1) in this process?

To investigate these aims, the main method used was the whole-cell patch-clamp recording of excitatory- and/or inhibitory postsynaptic currents (EPSCs, respectively IPSCs). We also used behavioral measurement of mechanical/thermal sensitivity and immunohistochemistry.

Our results have shown that: (I.) Direct functional interaction between TLR4 and TRPV1 receptors, in particular via PI3K signaling, play an important role in (*a*) PAC-induced increase of miniature EPSCs frequency in dorsal horn neurons, (*b*) in the modulation of TRPV1 sensitivity and tachyphylaxis of capsaicin-evoked responses, and (*c*) in the PAC-induced mechanical allodynia. All these PAC-induced changes have been prevented by PI3K blocker wortmannin. The TRPV1-dependent mechanism is also necessary to PAC-induced enhancement of c-Fos protein expression in the dorsal horn neurons. (II.) Our preliminary data clearly demonstrates that disinhibition occurs in a significant manner in all tested models of pain (PIPN, peripheral inflammation, and CCI). (III.) $Na_v1.7$ receptor blocker protoxin II significantly reduced aberrant activity induced by burn injury in the population of capsaicin-sensitive nociceptive spinal cord dorsal horn neurons in the rat. Finally, (IV.) we confirmed the hypothesis that 20:4-NAPE serves as a source for endogenous AEA synthesis in the spinal cord *in vitro*. Inhibitory effect

of 20:4-NAPE is mediated by CB_1-dependent mechanism. However, this CB_1-mediated analgesic effect of 20:4-NAPE, is under inflammatory conditions partly modified by an additional TRPV1-dependent mechanism.

Taking together, these data support the view that spinal nociceptive synaptic transmission is substantially influenced under pathological conditions, and that appropriate intervention and pharmacological treatment can help alleviate increased nociceptive transmission or pain-related behavior in animals.

Detailed understanding of these mechanisms is necessary for the improvement of pain therapy in the future.

Key words:

Pain, nociception, synaptic transmission, neuropathic pain, paclitaxel, TRPV1, capsaicin, TLR4, PI3K, $Na_v1.7$, CB_1, anandamide.

List of Abbreviations

$[\mathbf{Ca^{2+}}]_\mathbf{i}$	Intracellular Ca^{2+} concentration
20:4-NAPE	N-arachidonoylphosphatidylethanolamine
2-AG	2-arachidonoyl-glycerol
5-HT	Serotonin (5-hydroxytryptamine)
AEA	Anandamide (N-arachidonoylethanolamine)
AKAP	A-kinase Anchoring Protein
Akt/PKB	Protein Kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	Glutamatergic AMPA Receptor
ANOVA	Analysis of Variance
AP	Action Potential
AP5	2-amino-5-phosphonopentanoic acid (NMDA inhibitor)
ATF3	Nuclear Activating Transcription Factor
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
β-END	β-Endorphin
BIC	Bicuculline
BK	Bradykinin
BSA	Bovine Serum Albumine
CaM	Calmodulin
CaMKII	Ca^{2+}/Calmodulin Dependent Kkinase II
cAMP	Cyclic Adenosine Monophosphate
CAPS	Capsaicin
CB$_1$	Cannabinoid Receptor 1
CB$_2$	Cannabinoid Receptor 2
CCI	Chronic Constriction Injury
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Receptor for CCL2
CFA	Complete Freund's Adjuvant
CGRP	Calcitonin-Gene Related Peptide
CIPN	Chemotherapy-Induced Peripheral Neuropathy
C-M$_\mathbf{i}$H$_\mathbf{i}$	C-Mechano-insensitive, Heat-insensitive Fibers
CNQX	AMPA Receptor Blocker
CNS	Central Nervous System
CX3CL1	Fractalkine
CX3CR1	Fractalkine Receptor
Cx43	Connexin 43
CXCL1	Chemokine (C-X-C motif) Ligand 1
DA	Dopamine
DAMPs	Dangerous-Associated Molecular Patterns
DH	Dorsal Horn
DMSO	Dimethyl Sulfoxide
DRG	Dorsal Root Ganglia

eEPSC	Evoked Excitatory Postsynaptic Current
EP2	Receptor for Prostaglandine E_2
EPSC	Excitatory Postsynaptic Current
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
eV/eCBs	Endovanilloids/Endocannabinoids
eYFP	Enhanced Yellow Fluorescent Protein
EX-INs	Excitatatory Interneurons
GABA$_A$R	Ionotropic GABA$_A$ Receptor
GABA$_B$R	Metabotropic GABA$_B$ Receptor
GABA	γ-aminobutyric acid
GAD	Glutamate Decarboxilaze (types GAD65/67)
GAT(1–3)	GABA Transporters 1–3
GDNF	Glial Cell-Line Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GLAST	Glutamate-Aspartate Transporter
GLT1	Glutamate Transporter-1
GlyRs	Glycine Receptor
GlyT(1/2)	Neuronal Glycine Transporter (1/2)
HMGB1	High Mobility Group Box 1 Protein
HTMRs	High-threshold mechanoreceptors
ChR2	Channelrhodopsin 2
IB$_4$	α-D-galactosyl binding Lectin IB$_4$
IFN	Interferon (α/γ)
IGF-1	Insulin-Like Growth Factor 1
IL-1(α or β)	Interleukin-1 (α or β)
IN-INs	Inhibitory Interneurons
INs	Interneurons
IPSC	Inhibitory Postsynaptic Current
IR	Immunoreactivity
JNK	c-Jun N-Terminal Kinase
KCC2	Potassium Chloride Cotransporter 2
le-IPSC	Light-Evoked Inhibitory Postsynaptic Current
LPS	Lipopolysaccharide
LTD	Long-Term Depression
LTMRs	Low-Threshold Mechanoreceptors
LTP	Long-Term Potentiation
LY	LY-294002 hydrochloride
MAPK	Mitogen-Activated Protein Kinase
mEPSC	Miniature Excitatory Postsynaptic Current
mGluR	Metabotropic G-protein-coupled glutamate receptors
mIPSC	Miniature Inhibitory Postsynaptic Current
Mrgprd	Mas-related G-protein-coupled receptor D
MyD88	Myeloid Differentiation Primary Response Protein 88
NA	Noradrenaline

NADA	*N*-arachidonoyl-dopamine
NAPE	*N*-acylphosphatidylethanolamine (syn. 20:4-NAPE)
Na$_v$1.7	Voltage-Gated Sodium Channel type 1.7
NFκB	Nuclear Factor κB
NGF	Nerve Growth Factor
NK$_1$R	Neurokinin 1 receptor
NKCC1	Na$^+$-K$^+$-2Cl$^-$ cotransporter-1
NMDA	*N*-methyl-D-aspartate
NMDAR	Glutamatergic NMDA receptor
NO	Nitric Oxide
NON-N	Non-nociceptive Neurons
NRM	Nucleus Raphe Magnus
NS	Nociceptive Specific Neurons
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
OLDA	*N*-oleoyl-dopamine
P	Postnatal Day/Probability Value (in statistics)
P2X$_{3/4/7}$R	Purinergic receptors
P2Y$_{12}$R	Purinergic receptors
PAC	Paclitaxel
PAF	Primary Afferent Fibers
PAG	Periaqueductal Gray Matter
PAMPs	Pathogen-Associated Molecular Patterns
P-APS	Paclitaxel-Induced Acute Pain Syndrome
Pax2	Paired box gene 2 protein
PEA	Palmitoylethanolamide
pERK	Phosphorylated Extracellular Signal-Regulated Kinase
PGE$_2$	Prostaglandin E$_2$
PI3K	Phosphatidylinositol 3-Kinase
PIP$_2$	Phosphatidylinositol-4,5-bisphosphate
PIPN	Paclitaxel-Induced Peripheral Neuropathy
PKA	Protein Kinase A
PKC	Protein Kinase C
PNS	Peripheral Nervous System
PNs	Projection Neurons
PPR	Paired-Pulse Ratio
ProTxII	Protoxin II
PSNL	Partial Sciatic Nerve Ligation
PWL	Paw Withdrawal Latency
PWT	Paw Withdrawal Treshold
RA	Rapidly Adapting
ROS	Reactive Oxygen Species
RVM	Medullary Reticular Formation
SA	Slowly Adapting
SEM	Standard Error of Mean
sEPSC	Spontaneous Excitatory Postsynaptic Current

SFN	Small-Fiber Neuropathy
sIPSC	Spontaneous Inhibitory Postsynaptic Current
SNARE	Soluble NSF (*N*-ethylmaleimide-sensitive factor) Attachment Receptor
SNI	Spared Nerve Injury
SNL	Spinal Nerve Ligation
SNT	Sciatic Nerve Transection
SP	Substance P
STAURO	Staurosporine
STR	Strychnine
TGFβ	Transforming Growth Factor β
TIR	Toll/Interleukin-1 Receptor
TIRAP	TIR Domain-Containing Adaptor Protein
TLR4	Toll-Like Receptor 4
TLRs	Toll-Like Receptors
TNFα	Tumour Necrosis Factor α
TRIF	TIR domain-containing adaptor-inducing Interferon-β
TrkA/B	Tropomyosin receptor kinase A/B
TRP	Transient Receptor Potential
TRPA1	Transient Receptor Potential Ankyrin type 1
TRPM8	Transient Receptor Potential Melastatine type 8
TRPV1	Transient Receptor Potential Vanilloid type 1
TTX	Tetrodotoxin
VEGF	Vascular Endothelial Growth Factor
VGAT	Vesicular GABA/glycinetransporter
VGCCs	Voltage-Gated Calcium Channels
VGLUT	Vesicular Glutamate Transporter
VGSCs	Voltage-Gated Sodium Channels
WDR	Wide-Dynamic Range
WMN	Wortmannin

Obsah

Introduction

An understanding of pain mechanisms represents one of the oldest problems in the history of medicine. The difficulty lies in the fact that pain perception is a complex biopsychosocial phenomenon that arises from the interaction of multiple neuroanatomical and neurochemical systems with a number of cognitive and affective processes (Garland, 2012). An important step forward in the scientific characterization and understanding of pain has been taken by Sir CHARLES SCOTT SHERRINGTON, who defined the pain as the *"psychical adjunct of an imperative, protective reflex"* (Sherrington, 1906).

Pain is usually induced following exposure to several different classes of stimuli of noxious intensity (including heat, mechanical and chemical stimuli), which are detected by sensory neurons, called nociceptors (Sherrington, 1906). Although *acute nociceptive pain* is accompanied by unpleasant sensations, it serves a useful purpose for individuals. The ability to feel pain is essential for health because it forces individuals to take care of their bodies. The physiological significance of pain is most prominent when comparing normal individuals and individuals with a rare genetic disorder congenital analgesia. Individuals with congenital insensitivity to pain often die in childhood. They may bite their tongue or even break bones without knowing it (Berkovitch *et al.*, 1998; Cox *et al.*, 2006; Nagasako *et al.*, 2003).

However, there are many pathological conditions, such as *chronic* and *neuropathic pain*, in which pain loses its protective function and does not bring any benefit to the individual. As stated by the founder of the International Association for the Study of Pain, Dr. JOHN JOSEPH BONICA, already in 1953, *"pain in its late phases, when it becomes intractable, it no longer serves a useful purpose and then becomes, through its mental and physical effects, a destructive force"* (Bonica, 1953).

Despite the impressive progress of modern medicine and therapeutic methods, management of *chronic* and *neuropathic pain* represents still a significant problem. Underlying molecular mechanisms of chronic/neuropathic pain development remain to be fully clarified. Chronic pain often accompanies states such *chemotherapy-induced peripheral neuropathy*, *nerve injuries*, *burn injury* or *chronic inflammatory diseases* and highly affects patients' quality of life. Currently used analgesic approaches are often ineffective or inappropriate for use in these painful conditions and often have significant side effects. Therefore, there is a need for the development of new analgesics drugs and approaches to better control these pain states.

The aim of this Ph.D. project was to find and clarify some new mechanisms that are responsible for the development of these painful conditions. The main focus was on the mechanisms of modulation of spinal nociceptive processing, involved in the development of pathological pain

states, such as chemotherapy-induced peripheral neuropathy, which is a common adverse effect of chemotherapeutic drug paclitaxel (PAC). The main attention was paid to the sensitization of TRPV1 (Transient Receptor Potential Vanilloid type 1) channels through TLR4 (Toll-Like Receptor 4) signaling after paclitaxel treatment. Another issue we have studied was, how do different pain states, including PAC-induced peripheral neuropathy, acute peripheral inflammation and chronic constriction injury of sciatic nerve affect the inhibitory synaptic transmission in the spinal cord dorsal horn. We have also studied burn-injury induced spinal nociceptive processing, with focus on the role of voltage-gated sodium channel $Na_v1.7$ inhibition, as a potential target for pain management in burn injury patients. Part of the attention was also paid to the cannabinoid receptor CB_1- and TRPV1-mediated effect of endogenous lipid precursor of anandamide—20:4-NAPE, in inflammatory conditions. The experimental work was published in five original articles that are attached as an APPENDIX (p. 153).

Part I.

LITERATURE REVIEW

1. Pain Definition and Classification

The International Association for the Study of Pain (IASP) defines the pain as: *"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"* (Merskey & Bogduk, 1994)[1]. This definition clearly indicates that pain is not only a sensory process. In addition to the sensory component, pain has also a strong affective component, as well as a cognitive component involved in the anticipation of future harm (Garland, 2012). It is also important to mention the following facts: (*I.*) Pain is always subjective and therefore is difficult to measure it objectively; (*II.*) The inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment (Merskey & Bogduk, 1994).

The meaning of the term *"pain"* is very broad and is commonly used to denote all unpleasant and painful feelings, no matter how long they persist or which particular mechanisms are responsible for their origin.

There are several ways to classify pain. Based on the length of duration, we can classify pain as *acute*, *subchronic* and *chronic.*

ACUTE PAIN is caused by the phasic activation of nociceptors by potentially dangerous stimuli that exceed the physiological range. After processing at the spinal cord level, it evokes motor withdrawal and/or flight reaction. Acute pain is experienced immediately after noxious stimulation; its duration is short—in a range of seconds (Millan, 1999).

SUBCHRONIC PAIN lasts for several hours to days. It may be associated with the development of *allodynia*[2] and *hyperalgesia*[3]. It promotes the healing process as we take care to protect an injured body part. It often accompanies inflammation and it usually resolves upon tissue recovery (Millan, 1999).

CHRONIC PAIN persists beyond the usual healing course of an acute injury or disease (Bonica, 1990). It lasts months to years; often without obvious cause or it is disproportionately

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[1] Last updated version (December 14, 2017) of IASP pain terminology based on original Merskey & Bogduk (1994,) classification were used (available on-line: https://www.iasp-pain.org/terminology).

[2] ALLODYNIA is a pain due to a stimulus that does not normally provoke pain. It is important to recognize that allodynia involves a change in the quality of a sensation, whether tactile, thermal or of any other sort. The original modality is normally nonpainful, but the response is painful (Merskey & Bogduk, 1994). Allodynia is usually a consequence of central sensitization following nerve injury or inflammation when a threshold of postsynaptic dorsal horn neuron is reduced (Woolf, 2011).

[3] HYPERALGESIA reflects an increased pain response to a stimulus that normally provokes pain (Merskey & Bogduk, 1994). Hyperalgesia is a consequence of peripheral sensitization of nociceptors when the responsiveness is increased and prolonged (Woolf, 2011).

large considering to the intensity of the stimulus. It may involve a mix of both inflammatory and neuropathic component (Millan, 1999).

Acute and *subchronic pain* has "physiological" protective function. These pains usually occur as a symptom of noxious stimulation or due to an injury or illness. In contrast, chronic pain lacks this protective function. Therefore it can be described as "pathological" (Millan, 1999) and it is considered to be a disease in itself (Raffaeli & Arnaudo, 2017).

Based on the diverse mechanisms[4] of origin we can classify pain as *nociceptive, inflammatory, neuropathic* and *dysfunctional pain.*

NOCICEPTIVE PAIN is an important component of our defense system that protects our body. By the IASP terminology is *nociceptive pain* defined as a *"pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors".* This term is designed to contrast with neuropathic pain and is used to describe pain occurring with a normally functioning somatosensory nervous system to contrast with the abnormal function seen in neuropathic pain (Merskey & Bogduk, 1994). It is induced only by noxious stimuli that acting on a specialized high threshold sensory neurons. This threshold for eliciting pain is not fixed and can be shifted up or down. The shift in responsiveness is the consequence of neural plasticity, which may be adaptive or maladaptive (Scholz & Woolf, 2002).

INFLAMMATORY PAIN is a common symptom during infection, ischemia, tumor growth or autoimmune processes. It is induced by the release of proinflammatory mediators from damaged and inflammatory cells, such as cytokines, growth factors, purines, kinins, amines, etc. (Scholz & Woolf, 2002).

NEUROPATHIC PAIN is elicited and experienced independently of nociceptive neurons stimulation. It is caused typically by lesion or disease of the peripheral (PNS) or central nervous system (CNS). It is usually a chronic maladaptive condition, which often leads to persistent pain symptoms (Merskey & Bogduk, 1994; Scholz & Woolf, 2002). People with this pain condition may experience burning or shooting pain. A feeling of numbness and tingling or a loss of sensation is also a common symptom. Neuropathic pain may occur randomly, or it may be constant.

DYSFUNCTIONAL PAIN is maladaptive, similarly to neuropathic pain. However, in contrast to neuropathic pain, dysfunctional pain can occur also in conditions in which there is no such damage of the nervous system or inflammation. Conditions that evoke this type of pain include *fibromyalgia, irritable bowel syndrome, tension-type headache, temporomandibular joint disease, intestinal cystitis* and other syndromes in which there exists significant pain without noxious stimuli and no, or minimal peripheral inflammatory pathology (Woolf, 2010).

Another option, how to classify pain is according to the site of origin. Using this criterion, we can distinguish *somatic* and *visceral pain.* As a SOMATIC PAIN is referred pain, which arises

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[4] Some of these mechanisms are unique to one painful condition; others are present in multiple clinical symptoms. In some patients, a single mechanism may produce pain; in others, multiple mechanisms may be involved. The same symptom may be generated by a number of mechanisms. On the other hand, a single mechanism may potentially produce a number of diverse symptoms (Scholz & Woolf, 2002).

from the skin, muscles, mucous, skeleton and joints. Visceral Pain originates in the internal organs that are located inside the main body cavities. There are some features that make visceral pain unique and different from somatic pain. These features are: (*I.*) visceral pain is not evoked from all viscera (liver, kidney or lung parenchyma are not sensitive to pain); (*II.*) it is not linked to visceral injury; (*III.*) it is referred to other, often remote, locations; (*IV.*) it is diffuse and poorly localized; (*V.*) it is accompanied by exaggerated motor and autonomic reflexes. Visceral pain tends to be diffuse because of the organization of visceral nociceptive pathways in the central nervous system, particularly the absence of a separate visceral sensory pathway and the low proportion of visceral afferent (Cervero, 2009; Cervero & Laird, 1999). It is also important to mention that both somatic and visceral pain may be considered as nociceptive.

At the end of this overview of classification should be noted that in most clinical painful conditions, the mix of pain components is involved and simply and sharp classification of pain is not possible. For example, chronic pain involves usually both neuropathic and inflammatory component. Following tissue injury, inflammation produces inflammatory nociceptive pain. However, inflammatory mediators may cause damage to the neurons and induce neuropathic pain. On the other hand, the injury of the nervous system may induce inflammatory reaction and contribute to inflammatory pain via *neurogenic inflammation*[5].

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[5] The phenomenon of Neurogenic Inflammation refers to the inflammation that is produced through the release of neuropeptides and other inflammatory substances from the primary afferent fibers, especially of small diameter. It may produce peripheral sensitization as a consequence of antidromic activation/efferent function of nociceptive fibers (Matsuda *et al.*, 2018; Millan, 1999).

2. The Basic Mechanisms of Nociception and Pain Processing

2.1. Nociception or Pain?

By the IASP definition, nociception is *"the neural process of encoding noxious stimuli"*. Noxious stimulus is a stimulus that is damaging or threatens damage to normal tissues. These types of harmful stimuli are recognized by nociceptors, high threshold sensory receptors of the peripheral somatosensory nervous system, which are capable of transducing and encoding noxious stimuli (Merskey & Bogduk, 1994). Consequences of detection of noxious stimuli in the environment may be autonomic (e.g., elevated blood pressure and increased heart rate; Moltner *et al.*, 1990; Tousignant-Laflamme *et al.*, 2005) or behavioral (e.g., motor withdrawal reflex and nocifensive behavior; Merskey & Bogduk, 1994).

Activation of the nociceptors and nociceptive pathways by noxious stimuli is not a pain and not necessarily implied pain sensation; even though we may well appreciate that the pain has most often a proximate physical cause (Merskey & Bogduk, 1994). Unlike pain, nociception does not require cognitive and emotional processing in the brain, and therefore nociception cannot be described as an *"experience"*. A conscious feeling of pain occurs when a nociceptive stimulus is processed in the cortical regions of the brain. A scheme of anatomical distribution of pain and nociception is in FIGURE 2.1 (p. 10).

The proper functioning of the nociceptive system is essential to protect the body from tissue damage. However, under the pathological conditions (such as in inflammatory or neuropathic diseases), the nociceptive system can become sensitized. Then, pain can be felt after non-noxious stimulation and it can also turn into the disease (Zeilhofer, 2005).

2.2. Primary Afferent Sensory Neurons

All sensory systems, including nociception, must convert stimuli from the environment into electrochemical signals. In the case of olfaction or vision, primary sensory neurons detect only one type of stimulus (chemical odorants or photons). In this respect, nociception is unique. Individual primary nociceptive afferent neurons have the remarkable ability to distinguish a variety of physical and chemical stimuli, including those of a physical and a chemical origin. In comparison with other sensory systems, nociceptors are equipped with a diverse repertoire of transduction
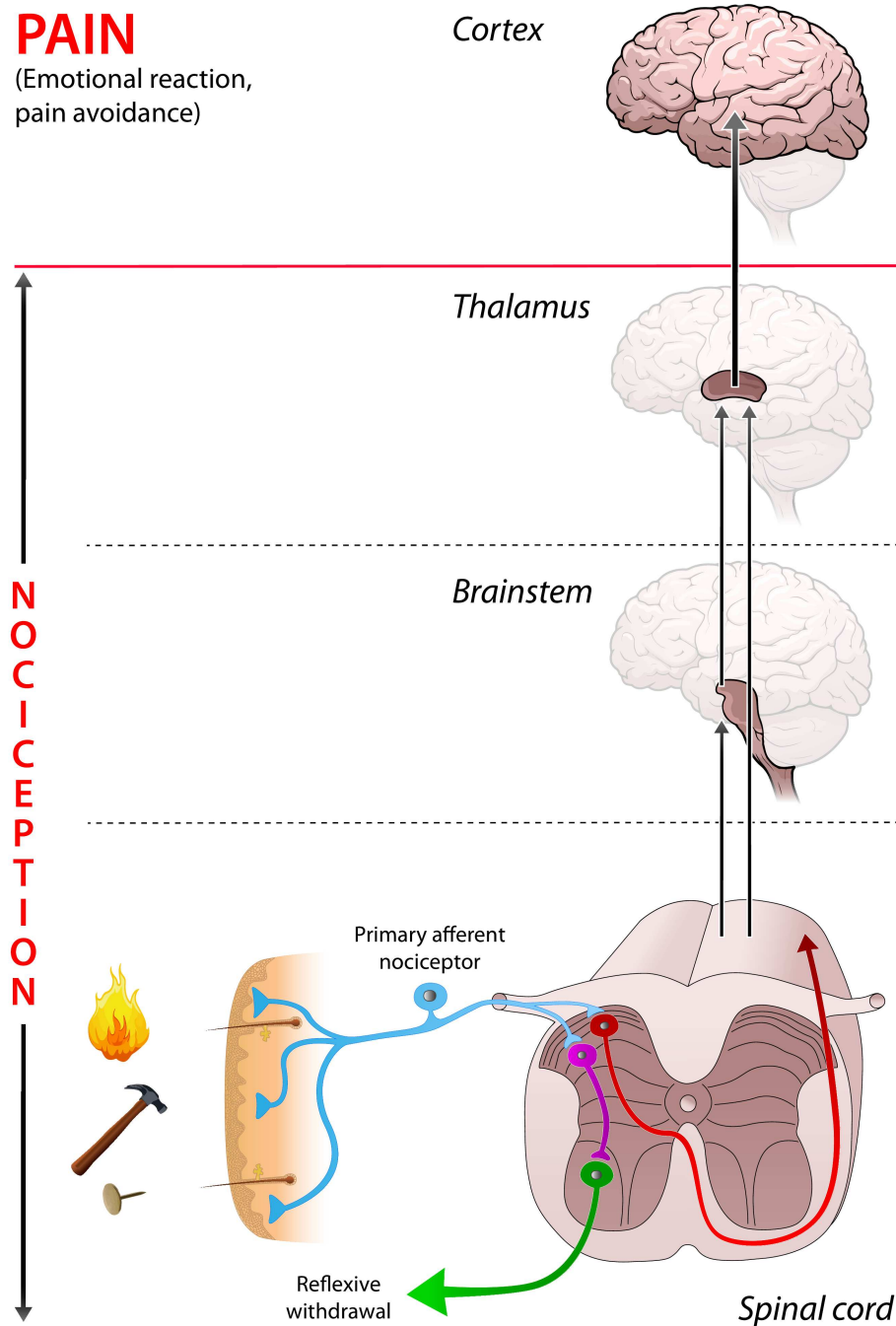
Figure 2.1.: **The scheme of anatomical distribution of nociception and pain.** This figure shows the major neuroanatomical structures that differentiate nociception and pain. The term nociception refers to the neural process that transmits information about noxious peripheral stimuli to the central nervous system. Nociceptive information is transduced by primary afferent neurons (nociceptors) to the dorsal horn of the spinal cord, brainstem, thalamus, and subcortical structures. Thalamus serves as the main relay center for sensory information to the cerebral cortex. Reflexive response and withdrawal after noxious stimulation represent a protective nociceptive mechanism that does not require conscious processing in cortical areas. For the experience of pain is fundamental processing of nociceptive information by thalamocortical networks (Figure adapted from Fig. 2-1; National Research Council Committee on Recognition and Alleviation of Pain in Laboratory Animals, 2009).

receptors. Some of those receptors are polymodal which means that a single receptor can transduce stimuli of a different nature simultaneously; e.g., Transient Receptor Potential Vanilloid type 1 (TRPV1) responds to chemical (capsaicin, acid) or physical (noxious heat) stimuli. These properties allow the single primary nociceptive sensory neuron to respond to a variety of noxious stimuli and enable it to integrate information and respond to complex changes in the environment (Julius & Basbaum, 2001).

All primary afferent neurons, including nociceptors, are from a morphological point of view pseudo-unipolar neurons (Kandel *et al.*, 2013). Cell bodies of these somatosensory neurons are located in *trigeminal* or *dorsal root ganglia* (DRG), where are surrounded by satellite glial cells that provide structural and metabolic support (Hanani, 2005). Pseudo-unipolar cells are variants of bipolar cells. During the development, the two processes of the embryonic bipolar cell fuse and emerge from the cell body as a single process with a "T-shaped" bifurcation that has two functionally distinct branches (Kandel *et al.*, 2013). Both of them function as axons; the *distal* one extends to the periphery, and forms the sensory endings in the skin, muscles, joints, and viscera; the *proximal* one forms synapses on a second-order neuron in the dorsal horn of the spinal cord or in the trigeminal *subnucleus caudalis* in the brainstem (Dubin & Patapoutian, 2010).

According to traditional classification, primary afferent fibers of sensory neurons can be in general classified into following groups (Aα, Aβ, Aδ, and C-fibers) based on the different morphological and physiological properties, such as their myelination, diameter, conduction velocity or threshold. A summary of the properties of these primary afferent fibers is shown in TABLE 2.1 (p. 12). It shows that the conduction velocity is directly correlated to the diameter of axons of sensory neurons and whether or not they are myelinated. The size of the cell bodies of DRG neurons also corresponds to the diameter of the PAF. Neurons with highly myelinated axons of large diameter (Aα/Aβ-fibers) have the largest diameter bodies. Aδ-fibers have cell bodies of medium-diameter and C-fibers without myeline shealth have small-diameter cell bodies (Olson *et al.*, 2016).

The sensory neurons with Aα-fibers innervate *muscle spindle receptors* and *Golgi tendon organ*, which signal muscle length and contractile force (Kandel *et al.*, 2013). Another three groups Aβ, Aδ and C-fibers innervating densely the skin. Slowly conducting C-fibers represent the majority of sensory neurons in the peripheral nervous system and the percentual representation of cutaneous fibers is following: C 70 %, Aβ 20 %, and Aβ 10 % (Dubin & Patapoutian, 2010; Millan, 1999).

The sensory neurons with Aβ-fibers innervate cutaneous low-threshold mechanoreceptors (LTMRs), among which are following: slowly adapting (SA) *Merkel cell-neurite complex* and *Ruffini endings*; and rapidly adapting (RA) *Meissner's corpuscles*, *Pacinian corpuscles*, and *Lanceolate Endings* (Olson *et al.*, 2016).

Sensory fibers of Aδ and C-fibers have in the periphery so-called *free nerve endings* that may be functionally associated with Schwann cells or keratinocytes (Lewin & Moshourab, 2004).

Table 2.1.: **Classification and comparison of properties of primary sensory afferent fibers** (Kandel *et al.*, 2013; Lewin & Moshourab, 2004; Millan, 1999).

Fiber class	Myelination	Fiber diameter (µm)	Conduction velocity (m.sec^{-1})	Mechanoreceptors types	Innervated structure	Modality
Aα	Thickly myelinated	12–20	Fast; 72–120	Ia	Muscle spindle	Limb proprioception
				Ib	Golgi tendon organ	Limb proprioception
Aβ	Thickly myelinated	6–12	Fast; 36–72	RAI-LTMRs	Meissner corpuscles	Touch (Movement)
				RAII-LTMRs	Pacinian corpuscles	Touch (Vibration)
				SAI-LTMRs	Merkel cell	Touch (Presure)
				SAII-LTMRs	Ruffini endings	Touch (Stretching of the skin)
Aδ	Thinly myelinated	1–6	Intermediate; 4–36	Aδ-HTMRs (Aδ-Nociceptors; Type I and II)	Free nerve endings	Termoreception, Nociception
				Aδ-LTMRs (D-Hair)	Hair follicles	Hair movement
C	Unmyelinated	0.2–1.5	Slow; 0.4–2.0	Polymodal C-fibers: C-MH (C-mechano-heat) C-MC (C-mechano-cold) C-MHC (C-mechano-heat-cold)	Free nerve endings	Termoreception, Nociception
				C-LTMRs	Free nerve endings	Touch (the pleasurable component of affectionate/erotic touch)
				C-M$_i$H$_i$ (C-Mechano insensitive, heat insensitive); Silent nociceptors	Free nerve endings	Litle or no mechanosensitivity under physiological conditions

Under normal physiological circumstances, only neurons with C and Aδ-fibers of small- and intermediate diameter serves as the high-threshold nociceptors, whereas neurons with Aβ fibers serves as LTMRs. Indeed, stimulation of large Aβ fibers can reduce pain, as occurs when they are activated by rubbing of the skin (Julius & Basbaum, 2001). However, peripheral tissue injury or damage of the nervous system is thought to lead to a reorganization of synaptic connections made by low-threshold mechanoreceptors in the spinal cord. Then, low-threshold neurons with Aβ fibers, which normally produce an only innocuous sensation like touch, can begin to produce pain to innocuous stimulation—allodynia. These changes may be one of the causes of neuropathic pain development (Lewin & Moshourab, 2004; Scholz & Woolf, 2002).

2.2.1. Primary Afferent Nociceptors

It was already mentioned that only C and Aδ-fibers transduce nociceptive information under the normal physiological conditions. We can distinguish two phases of somatic pain due to the different construction velocity of these fibers. The *"first"* phase, mediated by Aδ-fibers, is rapid, acute and sharp pain. The *"second"* phase is mediated by C-fibers and it is delayed more diffuse and dull pain. In contrast, for visceral pain is unique that there are no first (fast) and second (slow) components. Instead, visceral pain is often poorly localized, deep and dull (Cervero, 2009; Julius & Basbaum, 2001).

However, it is also important to mention that not every Aδ and C-fibers serves as nociceptors/high-threshold mechanoreceptors (HTMRs). Many types of Aδ and C-fibers have been identified and both of them are known also as low-threshold Aδ-LTMRs and C-LTMRs (Olson *et al.*, 2016).

Aδ-fibers

There are two main types of Aδ-fibers—Aδ-LTMRs, and Aδ-HTMRs. The first of them, Aδ-LTMRs are also known as D-hair cells. They form longitudinal lanceolate endings around hair follicles in the skin. Aδ-LTMRs have an extremely low mechanical threshold and have rapidly adapting responses (Djouhri, 2016; Olson *et al.*, 2016). Another type Aδ-HTMRs are nociceptive neurons responding to high-intensity stimuli and may be subdivided into two main classes. Type I respond both to mechanical and chemical stimuli but have a relatively high threshold to heat stimuli (~ 53 °C). However, if the heat stimulus is present for a longer period, they become sensitized and the mechanical and heat threshold decrease. And more importantly, these nociceptors become sensitized also after tissue injury (Basbaum *et al.*, 2009). Type II Aδ-HTMRs have a very high mechanical threshold, but much lower heat threshold (~ 43 °C). Type I mediates the first acute pain provoked by intense mechanical stimuli, whereas type II mediates the first pain response to noxious heat stimuli (Basbaum *et al.*, 2009; Julius & Basbaum, 2001). There are also differences in the morphology of the central axon endings Aδ-LTMRs and Aδ-HTMRs terminate in different spinal cord laminae, with the Aδ-HTMs terminating mainly in lamina I and V, whereas Aδ-LTMRs terminating mainly in lamina III and II (Djouhri, 2016).

C-fibers

Primary afferent neurons with C-fibers may be divided into three groups. Most of the unmyelinated C-fibers respond to both noxious mechanical and thermal stimuli and therefore they are so-called polymodal C-fibers. Polymodal nociceptors are classified according to the range of mechanical and thermal/cold stimuli that activate these fibers. Thus, we can distinguish the following groups of polymodal C-fiber nociceptors: (*I.*) C-mechano-heat (C-MH), (*II.*) C-mechano-cold (C-MC), and (*III.*) C-mechano-heat-cold (C-MHC; Lewin & Moshourab, 2004). In addition, two further groups of C-fibers exist in significant numbers. The first of them represents mechanosensitive C-LTMRs that may mediate the pleasurable component of affectionate

social touch (Olson *et al.*, 2016). The last group displaying no mechano- and heat-sensitivity under physiological conditions. Therefore, these C-mechano-insensitive, heat-insensitive nociceptors (C-M_iH_i) fibers have been sometimes called "silent" or "sleeping" nociceptors. However, upon sensitization with *algogens* (e.g., capsaicin, the hot compound of chili peppers, or inflammatory mediators such as bradykinin) they may become responsive to heat stimuli and tonic pressure. These "sleeping" C-M_iH_i fibers innervate all studied tissues (including human skin), but most of them innervate visceral organs or joints (Gebhart, 2000; Lewin & Moshourab, 2004).

Nociceptive neurons with primary afferent C-fibers can be subdivided also based on different biochemical and functional properties (Hunt & Rossi, 1985). Following neurogenesis, immature nociceptors differentiate in two distinct pathways that lead to the formation of two major subpopulations of nociceptors—*peptidergic* and *non-peptidergic*. These two sets of nociceptors express different types of receptors and ion channels and also innervate distinct peripheral and central targets (Woolf & Ma, 2007).

For the PEPTIDERGIC population of C-fiber nociceptors is characteristic expression and release of two neuropeptides, substance P^1, and calcitonin gene-related peptide (CGRP). They express also neurotrophin receptor TrkA that is the high-affinity tyrosine kinase receptor for nerve growth factor (NGF). NON-PEPTIDERGIC C-fiber nociceptors express the c-Ret neurotrophin receptor that is the target for glial-derived neurotrophic factor (GDNF), as well for neurturin and artemin. Non-peptidergic nociceptors express also purinergic receptor $P2X_3$, a specific subtype of adenosine triphosphate (ATP)-gated ion channel. These neurons can be labeled selectively with the α-D-galactosyl binding lectin IB_4 (Basbaum *et al.*, 2009; Julius & Basbaum, 2001; Snider & McMahon, 1998). A large population of isolecitin IB_4^+ neurons (~ 75 %) expresses also the Mas-related G-protein-coupled receptor D (Mrgprd). These Mrgprd-expressing nociceptors are required for the full expression of mechanical but not thermal nociception (Dong *et al.*, 2001). These two classes of C-fibers differentiate also in their central termination patterns. Thus, peptidergic nociceptors form synapses mostly with the lamina I and outer lamina II_o NK_1R-positive[2] projection neurons, whereas IB_4^+ nociceptors innervate mostly *substantia gelatinosa*/inner lamina II_i neurons, including radial, tonic central, transient central, vertical, and antenna cells (Snider & McMahon, 1998; Wang & Zylka, 2009).

Nociceptors can also be distinguished based on their differential expression of ion channels that mediates noxious stimuli, such heat (TRPV1), cold (TRPM8), many chemical irritants (TRPA1), and acidic milieu (ASICs; Julius & Basbaum, 2001). These functionally and molecularly heterogeneous classes of nociceptors associate with a specific function in the detection of distinct pain modalities (Basbaum *et al.*, 2009).

Recent advances in single-cell transcriptomics have brought new powerful tool for studying gene regulation at high resolution and for defining sensory neurons into distinct neuronal types

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[1] Not only C-fibers exhibited Substance P–like immunoreactivity (SP–LI). SP–LI was detected also in the population of Aδ nociceptors (Lawson *et al.*, 1997). Therefore, some Aδ-nociceptors may be called in literature also as peptidergic, similarly to C-fibers (Todd, 2010).

[2] Neurokinin 1 (NK_1R) receptor is a G-protein coupled receptor for substance P.

based on the different transcriptome (Thakur *et al.*, 2014; Usoskin *et al.*, 2015). It has been shown recently that DRG sensory neurons could be using single-cell RNA sequencing classified into *eleven types* in mouse—three distinct types of low-threshold mechanoreceptive neurons, two proprioceptive, and six principal types of thermosensitive, itch sensitive, type C low-threshold mechanosensitive and nociceptive neurons, with markedly different molecular and operational properties (Usoskin *et al.*, 2015).

2.3. Processing of Nociceptive Information in the Dorsal Horn

The spinal cord dorsal horn (DH) is the first site of synaptic processing in the pain pathway. In the DH are integrated both stimuli of exogenous origin, coming from the periphery, with stimuli of endogenous origin, which comes from visceral organs. Synaptic processing of nociceptive information, i.e., transmission of pain signals from the periphery to the brain is controlled by several mechanisms that can modulate it via: (*I.*) inhibitory control by descending pathways from supraspinal regions (More details in SECTION 2.4.2, p. 23), (*II.*) via the activity of Aβ-collaterals, and (*III.*) via spinal modulation by a variety of endogenous modulators (Steeds, 2013). Therefore, synaptic transmission between primary afferent neurons and secondary projection neurons in DH is not a fixed process. Rather, it is subject to the dynamic control by local interneurons, descending pro- and anti-nociceptive pathways and also chemical mediators released from neurons, non-neuronal glial and immunocompetent cells (Zeilhofer, 2005).

The principle fast excitatory neurotransmitter released in DH is excitatory amino acid L-glutamate. Synaptically released L-glutamate acts primarily on postsynaptic glutamate receptors, including ionotropic AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors), kainate receptors, and NMDA (*N*-Methyl-D-aspartic acid receptors), and also metabotropic G-protein-coupled glutamate receptors (mGluR; Coggeshall & Carlton, 1997; Erreger *et al.*, 2004; Zeilhofer, 2005).

There are also other pro-nociceptive modulators[3], including mediators such as neuropeptides substance P, neurokinin A and CGRP (Khawaja & Rogers, 1996; Sun *et al.*, 2004), ATP (Sawynok & Liu, 2003), cytokines and chemokines (e.g., tumor necrosis factor α (TNFα; Špicarová & Paleček, 2010), chemokine (C-C motif) ligand 2 (CCL2; Špicarova *et al.*, 2014a), chemokine (C-X-C motif) ligand 1 (CXCL1; Cao *et al.*, 2014), interleukin-1β (IL-1β; Donnerer & Liebmann, 2018), IL-1α (Kras *et al.*, 2014), IL-6 (Fang *et al.*, 2015)), nerve growth factors (e.g., NGF; Bonnington & McNaughton, 2003), brain derived neurotrophic factor (BDNF; Donnerer and Liebmann, 2018), GDNF (Fang *et al.*, 2003), nitric oxide (NO; Cury *et al.*, 2011), prostanoids and other metabolites of phospolipides (Kras *et al.*, 2014), neuropeptides bradykinin, galanin or cholecystokinin, and others.

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[3] Some of the mentioned modulators (e.g., and GDNF, NO, galanin) may have a dual effect on nociception. Depending on the situation and the site of action, they may be both pro- and anti-nociceptive.

Among the inhibitory substances involved in the modulation of nociception in DH are mainly inhibitory amino acids γ-aminobutyric acid (GABA) and glycine (Zeilhofer *et al.*, 2012) and endogenous opioid and cannabinoid systems (Steeds, 2013). The balance between excitatory and inhibitory mechanisms is absolutely essential to maintain normal perception. Disturbance of this balance, for example, due to disinhibition (loss/weakening of inhibitory transmission) or due to sensitization of excitatory component, can lead to allodynia, when light tactile stimuli are perceived as painful (Sivilotti & Woolf, 1994). Reducing the activity of inhibitory, GABAergic and glycinergic spinal circuits can contribute to the development of inflammatory and neuropathic pain much more than the increased sensitivity of sensory neurons themselves (Zeilhofer, 2005).

2.3.1. Laminar Organization of the Dorsal Horn

Neurons of the DH receive a highly organized somatosensory input from the periphery. In the presence of noxious stimuli, nociceptive input is conveyed by primary afferent neurons to *second-order neurons* in the DH of the spinal cord[4], where are located the first synapses on the *"pain pathway"* (Dubin & Patapoutian, 2010). Nociceptive information is in DH processed and modulated by local interneurons and by descending tracts before being transmitted to reflex circuits in deeper laminae, and to the brain (Gutierrez-Mecinas *et al.*, 2018). Cell bodies of second-order neurons are located in the grey matter of the spinal cord. The grey matter is divided into anatomically and electrophysiologically distinct laminae (FIGURE 2.2, p. 17). Laminar organization was originally described by BROR REXED in cat spinal cord and this scheme has since been applied to other species (Rexed, 1952). The DH is composed of lamina I (*marginal zone*), lamina II (*substantia gelatinosa*), laminae III and IV (*nucleus proprius*) and deep laminae V and VI (*deep layers*). Lamina VII forms the intermediate grey matter, whereas laminae VIII and XI comprise the medial and lateral ventral horn of the spinal cord. The last, lamina X is the area surrounding the central canal (*canalis centralis*; Millan, 1999).

The central endings of primary afferent neurons terminate with a specific distribution pattern that is determined by their functional class (FIGURE 2.2 B). In general, most of Aδ- and C-fibers nociceptors terminate in superficial laminae I and II$_o$. Aδ-fibers terminate to a lesser extent also in deeper laminae II$_o$, III, IV, V, and X. As well, a minor part of C-fiber nociceptors projects to laminae IV and X. In contrast, most of myelinated LTMRs with Aδ/β-fibers arborize in laminae II$_i$–V (Millan, 1999; Todd, 2010).

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[4] This is valid for nociceptive signaling from the body surface. The area of the face is innervated by the *trigeminal nerve* that forms synapses with second-order neurons located in the brainstem, in the trigeminal *subnucleus caudalis* (Dubin & Patapoutian, 2010).
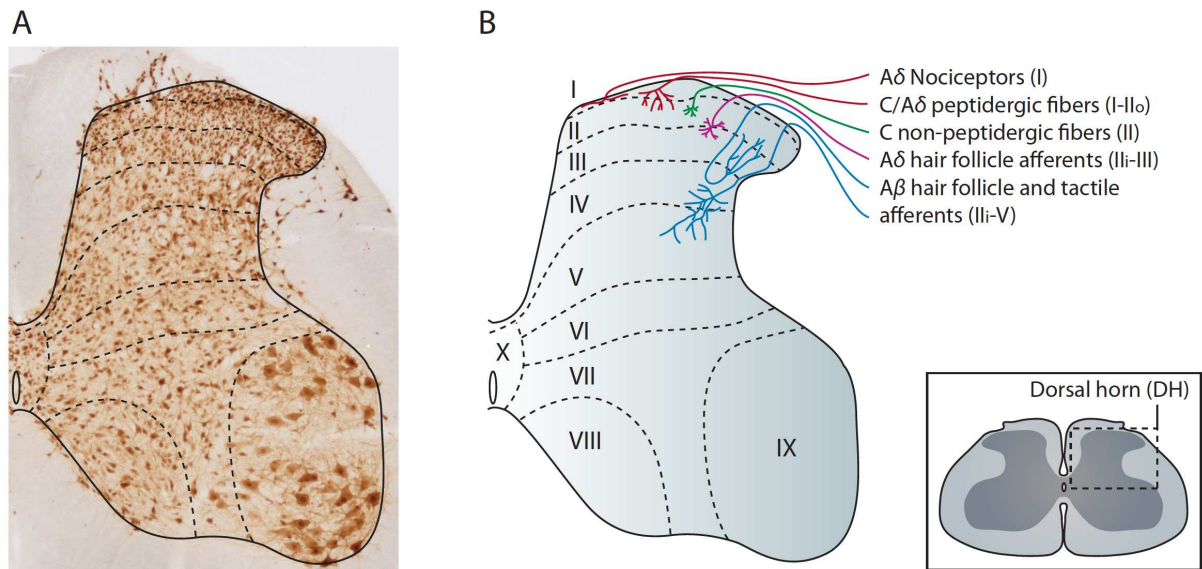
Figure 2.2.: **The scheme of the laminar organization of the spinal cord dorsal horn and primary afferent inputs.** The grey matter of spinal cord dorsal horn is divided into teen so-called *"Rexed laminae"* based on variation in the size and density of neurons. In the original work, BROR REXED (1952) described the laminar organization in the cat; this scheme has since been applied also to other species. **(A)** A transverse immunostained section of rat lumbar spinal cord, labeled with NeuN antibody that specifically labels neurons. The dashed lines show laminar boundaries. Lamina I (also known as the *marginal zone*) and lamina II (also known as the *substantia gelatinosa*) constitute the superficial dorsal horn and are characterized by the presence of numerous small neurons. Lamina II may be further subdivided into outer II_o and inner II_i parts. Lamina II_i has a lower density of neurons. The majority of the central endings of nociceptors terminate onto lamina I and II second order neurons. **(B)** The central terminations of the major primary afferent fibers types (excluding proprioceptors with $A\alpha$-fibers) are shown. Tactile $A\beta$-LTMRs innervate mainly neurons in lamina III–V, with some extension into lamina II_i; the precise arrangement depending on their function. $A\delta$-LTMRs (D-Hair) arborize on the border between lamina II and III, whereas $A\delta$-nociceptors innervate mainly in lamina I, with some branches in lamina V and X. Peptidergic primary afferents (which also include some $A\delta$-nociceptors, not only C-fibers) arborize mainly in lamina I and lamina II_o, with some terminals penetrating more deeply. Most non-peptidergic C-fibers terminate in the central part of lamina II (Reprinted from Todd, 2010).

2.3.2. Second-Order Neurons

The dorsal horn neurons represent functionally and morphologically heterogeneous population. There are several criteria for classification of second-order DH neurons.

The first criterion for the classification of the DH neurons is based on their *response characteristics*. Basically, three types of neurons may be identified within the DH: The first, *non-nociceptive* (NON-N) neurons are located predominantly in laminae II–IV, exceptionally in lamina I. NON-N neurons receive sensory input from Aβ-LTMRs. The second, *nociceptive specific* (NS) neurons are high-threshold nociceptors that are normally silent and activated exclusively by noxious stimuli, mediated by Aδ and C-fibers. Most of NS neurons are located in superficial laminae I and II$_\text{o}$, some of them are located also in deeper laminae V and VI. The last type is the so-called *Wide-Dynamic Range* (WDR) neurons. It is a population of neurons, located predominantly in lamina V, that receive a convergent input via direct/monosynaptic connection with Aβ and Aδ-fibers and also indirect/polysynaptic C-fiber mediated inputs. On WDR neurons converges coetaneous, muscle and visceral inputs and therefore they respond to a broad range of stimulus intensities (Basbaum *et al.*, 2009; Millan, 1999). The convergence of visceral and somatic nociceptive input on the same lamina V neuron provides one explanation for the phenomenon of *"referred pain,"* a condition in which pain of visceral origin is experienced as pain, originating on the body surface. The referred pain is a common symptom of *myocardial infarction* and *angina pectoris*, experienced as a deep pain in the chest and left arm (Kandel *et al.*, 2013).

Another way to divide DH neurons depends on the area of their output destination. DH neurons may be classified as follows: *interneurons*, *propriospinal neurons*, and *projection neurons*.

Interneurons

As Interneurons (INs) are defined neurons whose axons remain within the spinal cord and arborize locally. The vast majority (more than 90 %; Punnakkal *et al.*, 2014) of lamina I–III neurons are local INs. They play a key role in the integration, modulation, and inter- and intra-laminar transfer of information from primary afferent neurons. They are critically involved in normal sensory processing and in the development of pathological pain states. Two main classes of interneurons can be distinguished—excitatory (EX–INs) and inhibitory (IN–INs; Millan, 1999; Todd, 2010). Recent studies have shown that each of these groups can be subdivided into several neurochemically distinct populations. The EX–INs use amino acid L-glutamate as the main neurotransmitter, whereas IN–INs use GABA (γ-aminobutyric acid) and/or glycine as their main neurotransmitter(s). Their cell bodies can be immunohistochemically labeled with antibodies against these amino acids. In the rat, laminae I, II and III contain 25, 30 and 40 % of GABAergic neurons, respectively. Glycine immunoreactivity (IR) is present especially in lamina III neurons and in some neurons in laminae I and II. Glycine–IR is in laminae I–III significantly restricted to GABA–IR cells, which suggest that many INs co-release GABA and

glycine, whereas the others are purely GABAergic (Todd, 2010; Todd *et al.*, 1996). Recently has been shown that Pax2 (paired box gene 2) is selectively expressed by essentially all GABAergic neurons in laminae I–V and therefore antibody against to Pax2 may be used as a somatic marker of GABAergic INs in the rat DH (Larsson, 2017). In the DH of adult mice was Pax2 expression found in 93 % of GABAergic neurons and in 92 % of glycinergic (Punnakkal *et al.*, 2014). The axons of IN–INs can be labeled using antibodies against *glutamate decarboxylase* (GAD; the enzyme synthesizing GABA), the *vesicular GABA/glycine transporter* (VGAT) or *neuronal glycine transporter* (GLYT2). Using of these markers reveal a dense plexus of inhibitory axons in laminae I–III (Todd, 2010).

The excitatory dorsal horn neurons, respectively their axons in the DH can be labeled with an antibody against vesicular glutamate transporter 2 (VGLUT-2). In laminae I–III are located numerous VGLUT-2-positive synaptic boutons and most of them originate from local EX–INs. In contrast, VGLUT-1 is mainly present in primary afferents with large, CGRP-negative DRG neurons, and VGLUT-3 positive fibers probably have a supraspinal origin (Oliveira *et al.*, 2003). There are no available general immunohistochemical markers for the labeling of the cell bodies of all glutamatergic DH neurons. It is possible to identified EX-INs indirectly through the absence of staining for Pax2, and positive labeling with the pan-neuronal marker *NeuN* (Punnakkal *et al.*, 2014). This approach revealed $\sim$ 33 % presumed EX–INs in mice DH (Punnakkal *et al.*, 2014), whereas other sources refer about 60–70 % of the neuronal population in laminae I–III are EX-INs (Todd, 2015). There are several neurochemical markers that are found exclusively in EX–INs, including neuropeptides substance P, neurokinin B, somatostatin and neurotensin (Todd, 2010), the calcium-binding protein *calbindin* (Antal *et al.*, 1991) and *calretinin* (Albuquerque *et al.*, 1999), and the *γ-isoform of protein kinase C* (PKCγ; Polgar *et al.*, 1999). Among these, also other markers have been studied. For example, the calcium-binding protein *parvalbumin* that is increased under the inflammatory condition in a population of somatostatin-positive excitatory DH neurons (Sojka *et al.*, 2010). However, parvalbumin was found also in the population of GABAergic IN–INs (Antal *et al.*, 1991).

Among neurochemical differences, IN–INs and EX–INs also differ in some electrophysiological parameters. A team of Prof. HANS ULRICH ZEILHOFER using transgenic Gad67-eGFP, GlyT2-eGFP and vGLUT-eGFP mice strains showed that GABAergic–INs, glycinergic–INs, and EX–INs differ in some biophysical properties; significant differences were found in the rheobase[5], action potential (AP) threshold, and AP width (Punnakkal *et al.*, 2014). DH lamina II INs can also be classified according to the morphology of their dendritic trees. Four main classes of INs may be identified: *islet cells*, *central cells*, *vertical cells* and *radial cells* (Grudt & Perl, 2002). However, in case of EX–INs, only three types of dendritic tree morphologies (vertical, central and radial), but no islet cell-type morphology were found in vGLUT2-eGFP mice strain (Punnakkal *et al.*, 2014). Another criterion for INs classification is based on different firing pattern after current injection. However, the firing pattern shows often only little correlation

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[5] RHEOBASE is defined as a minimum current amplitude that evoked at least one action potential.

with morphology, and therefore there is a question of whether the morphological classes that have been identified in lamina II DH represent genuine functional population (Todd, 2010). A further limitation of this classification is that all morphological studies included a substantial population ($\sim$ 25–30 %) of unclassified cells (Punnakkal *et al.*, 2014; Todd, 2010).

Propriospinal neurons

In comparison with interneurons, Propriospinal Neurons arborize widely within the spinal cord and therefore play an integrative role in the communication between different segments and between the contralateral and ipsilateral part of DH (Millan, 1999). Although propriospinal neurons have long been known, little is known about their neurochemical phenotype or about the termination pattern of their axons. Recently was shown, using retrograde tracing, that around 30 % of neurons in laminae I–II of the lumbar L5 segment project at least five segments cranially (up to the segments L1–T13). Moreover, unilateral viral vector–mediated Cre recombinase–dependent anterograde tracing of the L5 segment in combination with immunohistochemistry provide evidence that ipsilateral *lateral spinal nucleus*, rather than superficial DH, is the main target for axons of propriospinal neurons. A dense cluster of axons was also found in the dorsal part of the lateral funiculus on the contralateral side, seen from L2 to C5 segment. Immunohistochemical analysis also showed that one-third of the propriospinal neurons are substance P–expressing excitatory neurons. In, contrast, propriospinal neurons lack markers of inhibitory neurons (Pax2) and excitatory neurons (VGLUT2 and PKCγ immunostaining; Gutierrez-Mecinas *et al.*, 2018).

Projection neurons

In contrast to interneurons and propriospinal neurons, only Projection Neurons (PNs) directly transmit nociceptive information to the supraspinal centers. PNs are found predominantly in laminae I and scattered throughout lamina III–V (Todd, 2010). Few of PNs may nevertheless be found in laminae II and X. PNs may be activated directly/monosynaptically by C, Aδ and Aβ fibers. EX–INs may activate PNs indirectly via polysynaptic activation by primary afferent fibers (Millan, 1999). Lamina I PNs are not a homogenous group. The majority ($\sim$ 80 %) of PNs express the NK$_1$R—the main target for substance P. Both laminae I and III NK$_1$R-expressing PNs are densely innervated by peptidergic C-fibers. Morphologically, based on the orientation and branching pattern of PNs dendrites, the lamina I PNs can be classified into three types: *fusiform*, *pyramidal* and *multipolar* PNs (Todd, 2010). However, not all PNs are NK$_1$R-positive; among them are so-called *"giant cells"* which can be recognized because of the very high density of excitatory and inhibitory synapses, coating their cell bodies. Both NK$_1$R-positive and giant cells express c-Fos protein, as a result of *c-fos* proto-oncogene activation, following noxious stimulation of different nature (Coggeshall, 2005; Kalynovska *et al.*, 2017). For many PNs (especially for lamina I PNs) is typical that their axons pass contralaterally through the midline to the ventrolateral white matter, forming the ascending *spinothalamic tract* (also known as a *ventrolateral* or *anterolateral* tract). Central terminals of these spinothalamic

tract–forming PNs terminates in the thalamic nuclei and forms a pathway, providing presumably a major source of nociceptive input to the brain (Todd, 2010; Todd, 2015).

2.4. Pain Pathways

2.4.1. Ascending Pain Pathways

Nociceptive information is after processing in the dorsal horn of the spinal cord transmitted by five major ascending pathways to the higher brain areas. The following tracts are involved: *spinothalamic*, *spinoreticular*, *spinomesencephalic*, *cervicothalamic* and *spinohypothalamic*. The *spinothalamic tract* represents the major ascending nociceptive pathway in the spinal cord. It includes the axons of nociception specific, thermosensitive and WDR neurons, with cell bodies in laminae I, V–VII of the dorsal horn (Kandel *et al.*, 2013).

The spinothalamic pathway includes two tracts that transmit the nociceptive information to several thalamic nuclei that serve as the main relay center of the central processing of nociceptive information (FIGURE 2.3). Two of the most important thalamic regions are the *lateral* and *medial nuclear groups*. The first tract—*lateral neospinothalamic tract* transmit nociceptive information to the lateral nuclear group. This group consists of the *ventroposterior lateral nucleus*, the *ventroposterior medial nucleus*, and the *ventroposterior inferior nucleus*. These nuclei receive inputs from nociceptive specific and WDR neurons laminae I and V. The second, *medial paleospinothalamic tract*, convey information to the *medial nuclear group* of thalamic nuclei. This group comprises the *central lateral nucleus* and the *intralaminar complex* of the thalamus. The major input to these two medial regions is from deeper laminae VII and VIII (Kandel *et al.*, 2013; Rokyta, 2012a; Steeds, 2013; Vaculín, 2012). Injury of the spinothalamic tract or thalamic nuclei causes a severe form of pain—CENTRAL PAIN (Kandel *et al.*, 2013; Millan, 1999).

Finally, the conscious feeling of pain occurs after the processing of nociceptive information in cortical regions. The following cortical areas have been shown to be involved in the processing of painful stimuli: primary somatosensory cortex, secondary somatosensory cortex and its vicinity in the parietal operculum, insular cortex, anterior cingulate cortex, and prefrontal cortex. These areas probably process different aspects of pain in parallel (Treede *et al.*, 1999).

Among ascending pathways and tracts, there are also descending pathways (mentioned in next SECTION 2.4.2), which play an absolutely indispensable role in the normal physiological processing of nociceptive information and pain experience.
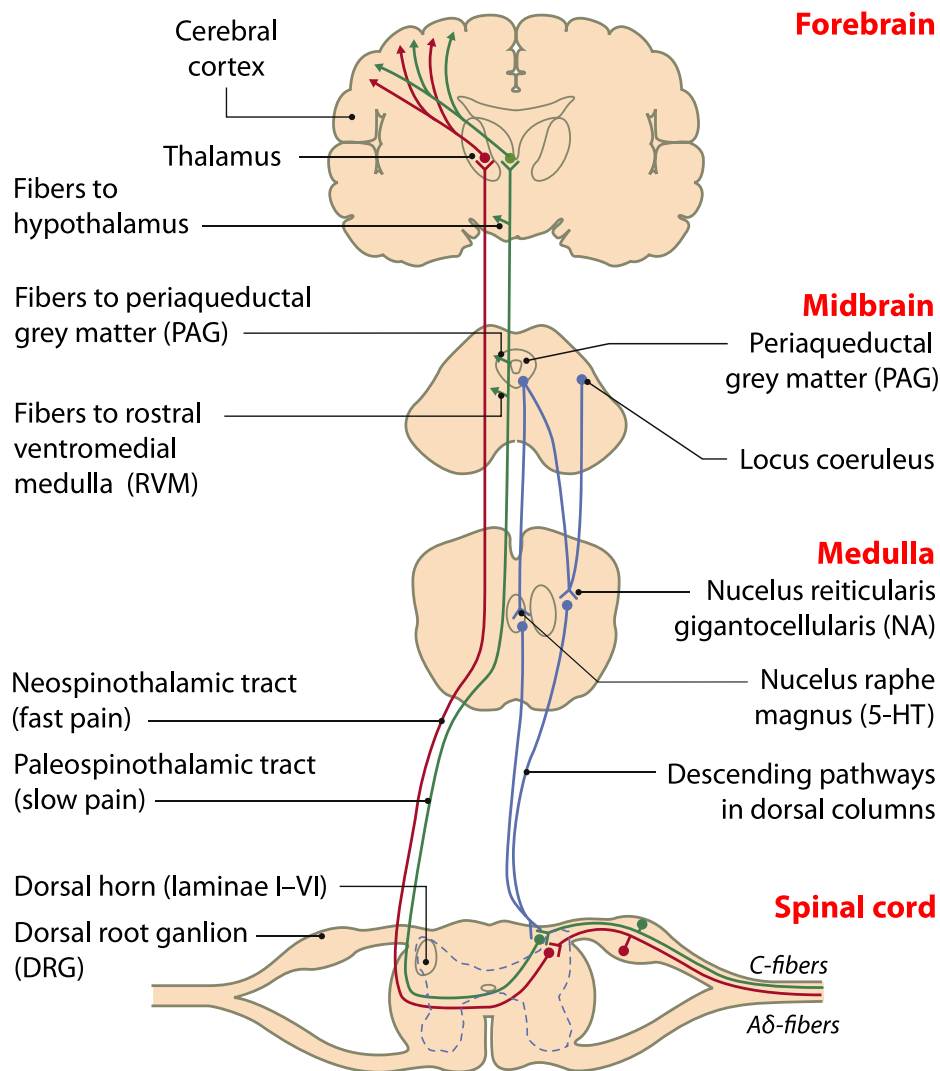
Figure 2.3.: **The scheme of ascending and descending pain pathways.** The *spinothalamic tract* represents the major ascending nociceptive pathway. After the noxious stimulation, "fast" component of pain mediated by Aδ-fibers, is transmitted by *lateral neospinothalamic tract* to the *lateral nuclear group* of the thalamus. In contrast, "slow" C-fibers mediated component is delivered to the *medial nuclear group* of thalamus via *medial paleospinothalamic tract*. Spinothalamic tract sends collaterals also into the *medullary reticular formation* (RVM), *periaqueductal gray matter* (PAG) and into the *hypothalamus*. Thalamus serves as a main central relay station and sends nociceptive information to final "pain processing" in cortical regions of the forebrain. Pain processing in the DH is modulated by descending pathways, which originate in several areas across the midbrain and medulla. PAG plays a pivotal role in the mechanism of descending inhibition of nociceptive processing. PAG neurons project down to the RVM, where is located serotonergic *nucleus raphe magnus* (NRM) and noradrenergic *nucleus reticularis gigantocellularis* that in turn project down to the spinal cord to modulate nociceptive synaptic transmission (Adapted from Steeds, 2013).

2.4.2. The Role of Descending Pathways in the Modulation of Spinal Nociceptive Processing

The descending pathways, projecting from the brain into the spinal cord DH, have a powerful modulatory influence on the outward transfer of somatosensory information from the spinal cord to the supraspinal areas.

According to the effect on the DH neurons, we can classify two types of descending modulation—namely *descending inhibition* and *descending facilitation*. The spinal pathways conveying descending facilitatory and inhibitory influences were found to be different. Both of them go through the white matter of the spinal cord; nevertheless, descending inhibitory influences were conveyed by *funiculus dorsolateralis medullae spinalis*, whereas facilitatory influences were conveyed by *funiculus ventrolateralis medullae spinalis* (Zhuo & Gebhart, 1997).

The main component of the descending system represents three monoamine-containing pathways—a *serotonergic*, *noradrenergic* and, to a lesser extent also a *dopaminergic system*, all projecting to the DH. However, besides the monoaminergic pathways, there is also a GABAergic and glycinergic descending pathway to the DH. The serotonergic and noradrenergic systems have the most important role in terms of controlling nociceptive transmission and pain mechanisms (Millan, 2002; Todd, 2010).

The SEROTONERGIC SYSTEM use serotonin (5-HT; 5-hydroxytryptamine) as a neurotransmitter. Serotonergic neurons originate mainly in the *nucleus raphe magnus* (NRM), which is part of the *rostral ventromedial medulla* (RVM; syn. *medullary reticular formation*) of the brain stem (Millan, 2002; Todd, 2010).

The NORADRENERGIC SYSTEM use noradrenaline (NA) as a neurotransmitter. The spinal cord is innervated by noradrenergic cells localized in the pontine nuclei *locus coeruleus* (A5 and A6 area) and in adjacent regions *locus subcoeruleus* (A7 area). Other noradrenergic neurons are located in the area of RVM, in the *nucleus reticularis gigantocelulularis* (Millan, 2002; Steeds, 2013; Todd, 2015).

The neurons of DOPAMINERGIC SYSTEM use dopamine (DA) as a neurotransmitter. The main source of dopamine within the brain represents neurons located in the *ventral tegmental area* and *substantia nigra pars compacta* of the midbrain. However, only a minor component of dopaminergic innervations of the spinal cord may originate in *substancia nigra* and in *paraventricular nucleus* of the hypothalamus, but the main source of dopamine within the spinal cord is mainly from the posterior periventricular nucleus (A11 area) of the hypothalamus (Millan, 2002; Sharples *et al.*, 2014).

There are also some non-monoaminergic pathways, involved in descending modulation. Descending projections of GABAERGIC/GLYCINERGIC SYSTEM originate in the RVM and arborizing throughout the DH (Antal *et al.*, 1996).

Essentially all of the components of descending control, mentioned above, are under the control of *periaqueductal grey matter* (PAG), which is considered to be the main control center of descending modulation. PAG is an area around central aqueduct in the midbrain

of the brainstem. PAG is under control of cortex, amygdala, and hypothalamus. Direct links form PAG to serotonergic and non-serotonergic neurons of the RVM, as well as to noradrenergic nuclei of the medulla. Direct projections to the DH from PAG are very sparse. PAG, together with RVM is the central substrate for actions of opioid drugs. PAG plays a key role also in the mechanism of endogenous analgesia (Millan, 2002; Steeds, 2013).

It was mentioned above that both descending inhibition and descending facilitation must be recognized. However, there is no absolute anatomical separation of substrates subserving these processes; both inhibitory and facilitatory pathways may originate in the same brain structures. It was known that the stimulation of a single supraspinal structure (involved in descending control, e.g., NRM or RVM) might via different mechanism trigger both descending facilitation and descending inhibition. As a further example of the complexity and sophistication of descending controls—a single neurotransmitter (5-HT, NA, DA) may, via divergent signaling expressed by different receptor types, concomitantly promote and suppress nociceptive transmission. Moreover, under the supraspinal descending control are both EX-INs and IN–INs, PNs and also central endings of primary afferent fibers with a different repertoire of receptors (Millan, 2002).

3. Neuropatic Pain

There are many clinical diseases and syndromes—e.g., Diabetes, Cancer or Chemotherapy-Induced Neuropathy) accompanied by the development of *neuropathic pain as a secondary symptom.* However, unlike the *nociceptive* or *inflammatory pain* (mentioned in Chapter 1, p. 5), *neuropathic pain* lacks its physiological and protective role. In contrast, neuropathic pain is maladaptive and occurs independently of nociceptor stimulation. The primary cause of neuropathic pain is an abnormal function of the nervous system due to damage or disease of the PNS and/or CNS. Therefore, neuropathic pain should be considered to be a disease rather than just a symptom. Chronic pain of neuropathic origin represents one of the most common reasons for hospital visits (Raffaeli & Arnaudo, 2017; Ueda, 2008).

According to primary insult to the PNS or CNS, the *clinical classification* of neuropathic pain differentiates between *neuralgias of peripheral nerve* (e.g., trigeminal neuralgia) and *central neuropathic pain* (e.g., thalamic syndrome). However, the peripheral or central site of underlying pathophysiology can no longer be used as a discriminant for neuropathic pain, because both peripheral and central mechanisms may be involved in the development of neuropathic pain pathophysiology. For example, after nerve injury of peripheral nerve, pain signals originate first from the axonal site of the lesion, but over the time, other parts of the injured sensory neuron (soma in DRG) and even the postsynaptic DH neurons and higher supraspinal mechanisms may contribute (Zimmermann, 2001).

More useful is a *phenomenological classification* relates to the type of damage or related pathophysiology causing a painful neuropathic disorder. Based on this perspective, we can distinguish: (*I.*) *Mechanical nerve injury* (e.g., carpal tunnel syndrome, vertebral disk herniation); (*II.*) *Metabolic diseases* (e.g., diabetic poly-neuropathy); (*III.*) *Neurotropic viral diseases* (e.g., herpes zoster virus or human immunodeficient virus (HIV) disease); (*IV.*) *Neurotoxicity* (e.g., side effects of chemotherapy of cancer (It will be mentioned in detail in Chapter 6. Chemotherapy-Induced Peripheral Neuropathy, p. 47) or tuberculosis); (*V.*) *Inflammatory and/or immunologic disorders* (e.g., multiple sclerosis); (*VI.*) *Nervous system focal ischemia* (e.g., thalamic syndrome; deafferentation pain syndrome (anesthesia dolorosa[1]); and (*VII.*) *Multiple neurotransmitter system dysfunction* (e.g., complex regional pain syndrome—CRPS; Zimmerman, 2001).

Neuropathic pain is characterized by the presence of stimulus-independent persistent pain and by an abnormal sensory perception of pain, such as *allodynia* (pain evoked by innocuous

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[1] Anesthesia Dolorosa is severe pain perceived in an area that is completely insensitive to touch and other types of stimuli.

Table 3.1.: **An overview of the basic peripheral and central mechanisms/phenomena that may contribute to the development of neuropathic pain** (Bridges *et al.*, 2001; Woolf, 2004; Rokyta, 2012b; Ueda, 2008).

Peripheral mechanisms contributing to the development of neuropathic pain:

❍ *Ectopic and spontaneous activity* (i.e., generating of action potentials independently of stimulation)

❍ *Ephaptic conduction* (a form of non-synaptic interaction/communication between unmyelinated collaterals)

❍ *Collateral sprouting* (sprouting of collateral fibers from sensory axons of the injured nerve into the denervated area—e.g., skin)

❍ *Demyelination of injured Aβ and Aδ-fibers* (reduction of the myelin sheath of neurons leads to the reduction in conduction ability and cause a deficiency in sensation; it may be accompanied with sprouting and ephaptic conduction)

❍ *Alterations in ion channel expression*

❍ *Change in the phenotype of DRG neurons* (injury of peripheral nerve induce synthesis of substance P and BDNF in A-fibers, although these substances are normally expressed only in peptidergic C-fibers)

❍ *Sprouting of sympathetic neurons into the DRG*

❍ *Sensitization of nociceptors*

❍ *Wallerian degeneration* (i.e., nerve injury-induced degeneration of the distal part of the axon)

Central mechanisms contributing to the development of neuropathic pain:

❍ *Central sensitization / long term potentiation* (neuroplastic changes leading to an increase in synaptic strength, i.e., synaptic transmission efficiency, for example in the DH)

❍ *Spinal reorganization* (changes in the localization of nociceptive central terminals—e.g., sprouting of Aβ-fibers into the lamina II after peripheral axotomy)

❍ *Cortical reorganization* (it is involved in phantom limb pain syndrome)

❍ *Changes in inhibitory pathways/disinhibition* (Decrease in GABAergic inhibitory tone)

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tactile stimulation) and *hyperalgesia* (increased pain sensation evoked by the mild noxious stimulus; Merskey & Bogduk, 1994; Ueda, 2008).

There are multiple mechanisms contributing to the development and maintenance of neuropathic pain syndrome. These include changes both at the cellular and at the molecular level it the PNS, spinal cord and brain (especially in the brainstem). Some of these changes are transient, some require the presence of peripheral pathology for their maintenance, and others may produce persistent and/or autonomous changes in the function of the nervous system. The etiological factors responsible for driving the mechanisms are not disease-specific and multiple mechanisms may coexist in a single given patient (Bridges *et al.*, 2001; Woolf, 2004). For a brief overview of the basic peripheral and central mechanisms responsible for the development of neuropathic pain, see TABLE 3.1.

3.1. Animal Models of the Nerve Injury-Induced Neuropathic Pain

The management of neuropathic pain represents a significant problem and currently available analgesic drugs often not adequately treat patients. In this section, the main animal models of nerve injury-induced neuropathic pain will be shortly mentioned. Animal models play in research a pivotal role because they provide primary systems for the preclinical study of pain. Numerous animal models have been developed to simulate human peripheral neuropathic conditions. Specific injury of the nervous system (most often based on procedures at or near sciatic nerves) in experimental animals may induce behavioral changes that are generally accepted as a model of human neuropathic pain (Wang & Wang, 2003). An overview of commonly used animal models of peripheral nerve injury is shown in FIGURE 3.1 (p. 28) and discussed below.

Total SCIATIC NERVE TRANSECTION (SNT or axotomy) or ligation has been reported as a model of phantom pain after amputation of the limb. This injury induces the formation of the proximal nerve stump, similarly to amputation, and degeneration of the distal part of the nerve. Animals expressed spontaneous pain and so-called *"autotomy"*, which describe self-attack behavior and mutilation of the deafferentiated limb (Wall *et al.*, 1979). The disadvantage of this model is the impossibility of quantification of typical symptoms of neuropathic pain—allodynia and hyperalgesia (Franěk, 2006).

Hence, other models based on partial sciatic nerve damage were designed—e.g., CHRONIC CONSTRICTION INJURY (CCI) model (originally, four loosely tightened *chromic gut* ligatures were used (Bennett & Xie, 1988). Another method to induced CCI uses fixed-diameter polyethylene cuffs applied to the sciatic nerve (Mosconi & Kruger, 1996).

PARTIAL SCIATIC NERVE LIGATION (PSNL) model procedure involves the ligation of $\sim 1/3$–$1/2$ thickness of the sciatic nerve (Seltzer *et al.*, 1990).

SPINAL NERVE LIGATION model (SNL) uses the ligation of both the L5 and L6 spinal nerves is used in rats, and only L5 spinal nerve is ligated in murine SNL model (Kim & Chung, 1992; Wang & Wang, 2003).

SPARED NERVE INJURY (SNI) model involves the lesion of two of three terminal branches of the sciatic nerve—*nervus tibialis* and *nervus peroneus communis* (Decosterd & Woolf, 2000).

Finally, CHRONIC CONSTRICTION OF DORSAL ROOT GANGLION (CCD) model is produced (in adult rats) by implanting a stainless steel rod unilaterally into the intervertebral foramen (foramina invertebralia), one rod at L4 and another at L5 (Song *et al.*, 1999). These models, starting with CCI and ending CCD, are characterized by spontaneous pain behavior and by the development of hypersensitivity to mechano/heat/cold stimulation (allodynia and/or hyperalgesia can be developed in varying degrees).
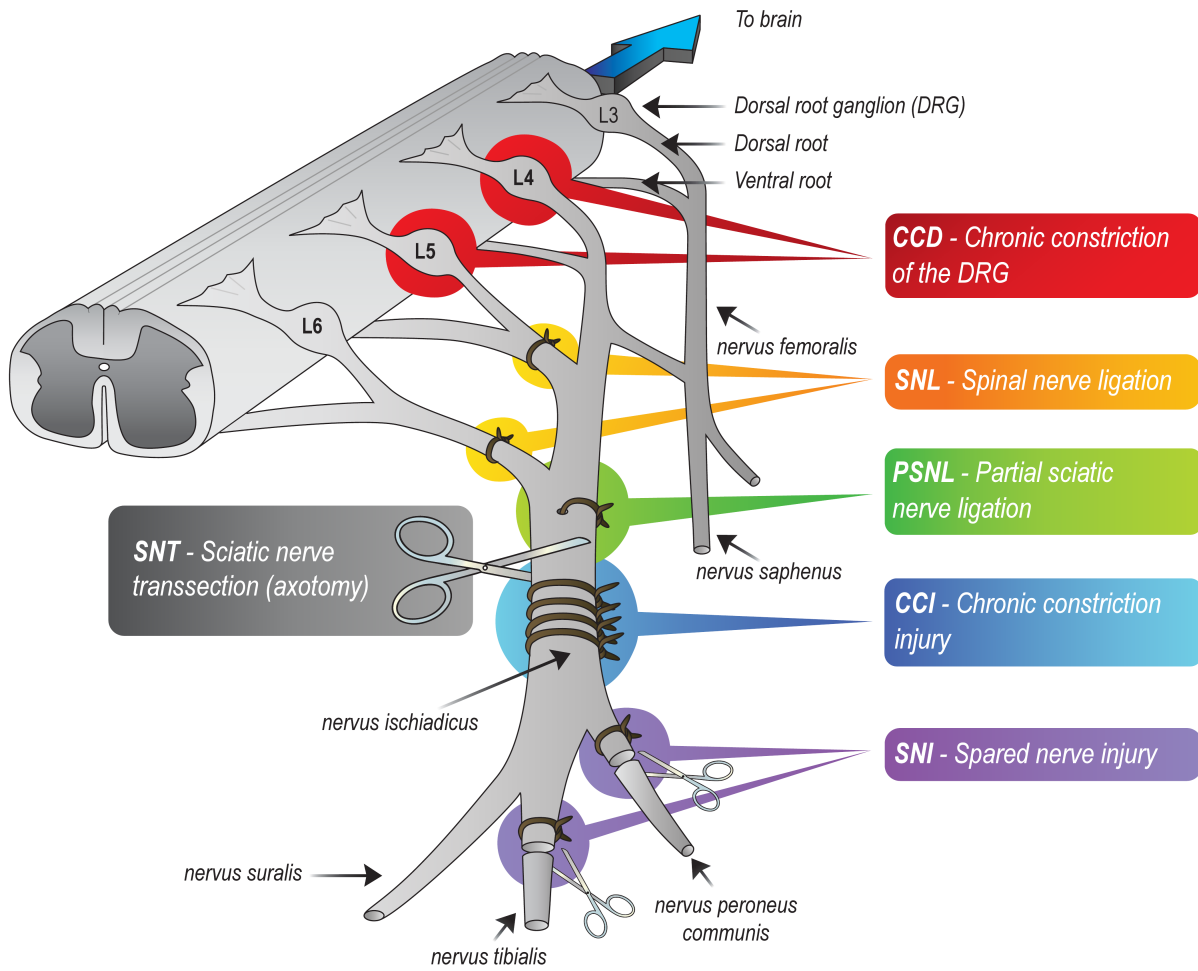
Figure 3.1.: **Animal models of the peripheral nerve injury** (Figure adapted from Adámek, 2014, Diploma Thesis).

4. Inflammation and Pain Plasticity

ACUTE INFLAMMATION (from Latin: *inflammatio*) is a biological response of vascular tissue to harmful stimuli, such as tissue injury, chemical irritants and/or infection by pathogens. It is characterized by five typical signs: *rubor* (redness), *calor* (increased heat), *tumor* (swelling), *dolor* (pain) and *functio lessa* (loss of function; Ji *et al.*, 2016; Winter *et al.*, 1962). Acute inflammation is a protective response of an organism and acute nociceptive pain is a cardinal feature of inflammation. Acute inflammation is a result of the activation of the innate immune cascade, including plasma extravasation and infiltration of immune cells, including macrophages, neutrophils, and T-cells into the damaged part of body tissue. The infiltrated immune cells and resident cells such as mast cells, macrophages, platelets, endothelial cells, fibroblasts, and keratinocytes, together with damaged cells of the injured tissue, release several inflammatory mediators, collectively referred to as the *"inflammatory soup"*. This "soup" including wide range of signaling molecules, e.g., neurotransmitters (glutamate, serotonin (5-HT), histamine), peptides (bradykinin, substance P, CGRP), eicosanoids and related peptides (prostaglandins (e.g. PGE_2), thromboxanes, leukotrienes, endocannabinoids—such as anandamide[1], extracellular proteases, ATP, NGF, many proinflammatory cytokines ($TNF\alpha$, IL-1β, IL-6) and chemokines (CCL2, CXCL1). The majority of known proinflammatory mediators may act on their respective receptors on peripheral endings of nociceptive neurons that innervate injured skin, muscles, or joint tissue. Activation of these receptors induces pain sensation and peripheral sensitization, which may help to protect damaged tissue during the healing process (Basbaum *et al.*, 2009; Ji *et al.*, 2016; Ji *et al.*, 2014).

In contrast to acute inflammation, CHRONIC INFLAMMATION is not beneficial for the organism and is rather harmful. Chronic inflammation often accompanies diseases such as *atherosclerosis*, *rheumatoid arthritis*, *periodontitis*, and even *cancer*. It is still uncertain whether chronic inflammation is also as necessary for the maintenance of chronic pain as acute inflammation is critical for driving acute pain (Ji *et al.*, 2016; Ji *et al.*, 2014).

Another term related to inflammation, NEUROGENIC INFLAMMATION resulting from nociceptor activation. It may be caused by intradermal administration of various irritants, such as capsaicin, which activates TRPV1, or mustard oil, which activates transient receptor potential ankyrin 1 (TRPA1; Matsuda *et al.*, 2018) or by antidromic activation of nociceptive fibers (Millan, 1999). Neurogenic inflammation is a characteristic feature of C-fiber nociceptors that release neuropeptides such as substance P, CGRP and prostanoids. These agents facilitate

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[1] Endocannabinoid anandamide (*N*-arachidonoylethanolamine; AEA) is an endogenous agonist of vanilloid TRPV1 and cannabinoid CB_1 receptor; for more information see SECTION 5.5, page 45

rapid plasma extravasation and edema, which occur faster than that of immune cell infiltration. Neurogenic inflammation contributes to pain conditions such as a headache, migraine, and inflammatory diseases including asthma and psoriasis (Ji *et al.*, 2018; Matsuda *et al.*, 2018). Neurogenic inflammation can occur not only in peripheral tissues but also in the CNS (Xanthos & Sandkuhler, 2014).

Neuroinflammation refers to the inflammatory process within the PNS and CNS. Neuroinflammation has a key role in the development of chronic pain and, as well, it is an underlying cause of several neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease or multiple sclerosis). Despite that, there are clear differences between neuroinflammation in neurodegenerative diseases and in chronic pain following peripheral injury. Neuroinflammation in neurodegenerative diseases and, e.g., after spinal cord injury is a result of direct damage of the CNS and it may cause further neuronal degeneration. In chronic pain states, both *neuropathic* and *inflammatory origin*, the neuroinflammation is often induced by peripheral damage and by excessive activity of primary nociceptive neurons (Ji *et al.*, 2014).

The key role in the development and maintenance of chronic pain states play Neuronal and Synaptic Plasticity—the capacity of neurons to change their function, chemical profile, or structure. Neural plasticity may be activation-dependent, or it may be induced via (*I.*) *modulation* and (*II.*) *modification of the pain system* (Woolf & Salter, 2000).

Modulation of nociceptive transmission may occur both in the periphery and in the CNS. The major mechanisms responsible for modulation are (*a*) phosphorylation of receptor and/or ion channels, or associated signaling molecules and regulatory proteins and (*b*) alterations in intrinsic properties and/or cell-surface expression of ion channels in primary nociceptive and also in DH neurons. Modulation of peripheral terminals leads to increased excitability that reduces the amount of depolarization required to initiate the action potential discharge and contributes to *peripheral sensitization* (Woolf & Salter, 2000).

Modification of pain system/transmission is mediated by alterations in gene expression, loss or weakening of inhibitory interneuron-mediated control (disinhibition), and by the establishment of aberrant excitatory synaptic activity (Sivilotti & Woolf, 1994; Woolf & Salter, 2000).

Peripheral sensitization, a *phenomenon of neuronal plasticity*, is induced by the sensitizing agents include for example bradykinin (Wang *et al.*, 2006), PGE_2 (Ma *et al.*, 2017), 5-HT (Rueff & Dray, 1992), ATP (Fabbretti, 2013) and NGF (Stein *et al.*, 2006; Zhu & Oxford, 2007) released during tissue injury or by inflammatory cells. These agents may via activation of G-protein coupled and tyrosine-kinase receptors activate intracellular pathways including several kinases, e.g., protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent kinase II (CaMKII) or phosphatidylinositol 3-kinase (PI3K). Activation of these kinases subsequently modulates the function of ion channels and receptors important in nociceptive transduction, e.g., tetrodotoxin (TTX)-resistant sodium channels and TRP channels (Woolf & Salter, 2000; Zhang *et al.*, 2011; Zhu & Oxford, 2007; Zhuang *et al.*, 2004).

CENTRAL SENSITIZATION, which can be understood as a *phenomenon of synaptic plasticity*, is by the official IASP definition *"increased responsiveness of nociceptive neurons in the CNS to their normal or subthreshold afferent input"* (Merskey & Bogduk, 1994). In the context or pain, central sensitization is emerging on synapses between primary nociceptive neurons and second-order DH neurons and mostly occur in response to increased activity of nociceptors (peripheral sensitization) caused by inflammation or nerve injury. Modulation leading to central sensitization involves activation of intracellular cascades that facilitate excitatory synaptic transmission, depress inhibitory control and thereby amplify responses to both noxious and non-noxious stimuli (Ji *et al*, 2018; Woolf & Salter, 2000).

An underlying mechanism of excessive excitatory transmission during central sensitization is similar to the phenomenon of long-term potentiation (LTP). It can be driven by the excessive activation of NMDA receptors resulting in an increase in the intracellular concentration of Ca^{2+} ions in the DH neurons. Increased Ca^{2+} level triggers Ca^{2+}-dependent molecular effectors of several signaling pathways including kinases such as PKC, PKA, CaMKII and mitogen-activated protein kinases (MAPKs), e.g., extracellular signal-regulated kinase (ERK1/2) or p38 MAPK. These kinases participate during the *early phase* of central sensitization on the modulation of glutamate NMDA and AMPA receptors. Phosphorylation of these receptors causes changes of activation kinetics and induces an increase in their conductivity. Moreover, receptor phosphorylation also regulates transfer and incorporation of AMPA receptors to the plasma membrane. During the *late phase*, activation of Ca^{2+}-dependent signaling produces activation of several transcription factors and changes in gene expression that strengthens central sensitization (Woolf, 2011; Woolf & Salter, 2000).

A critical role in the development of central sensitization also plays *disinhibition*—a reduction in the activity of GABAergic and glycinergic neurons and receptors. Moreover, disinhibition plays probably a more important role in the inflammatory and neuropathic pain than direct amplified excitation (Zeilhofer, 2005). The role of inhibitory control in the synaptic transmission processing will be discussed in CHAPTER 10 (p. 65). The loss of inhibitory control together with the increase in the membrane excitability and synaptic efficacy may "uncover" normally ineffective synapses, e.g., inputs from $A\beta$-LTMRs that can converge with nociceptive fibers to WDR projection neurons and activate the pain circuits and produce *secondary hyperalgesia*, i.e., increased pain sensitivity that occurs in the surrounding undamaged area of tissue injury (Woolf, 2011).

Accumulating evidence suggests that central sensitization is also driven by neuroinflammation in the PNS and CNS. A characteristic feature of neuroinflammation is the activation of glial cells, such as astrocytes and microglia, and infiltration of immune cells into the spinal cord and brain (Ji *et al.*, 2018). The role of non-neuronal cells in the pain processing and neuroinflammation will be discussed in the next SECTION 4.1 (p. 32).

When plasticity, for example, peripheral sensitization, facilitates protective reflexes, e.g., increased care for the injured tissue due to increased sensitivity to non-noxious temperature

or touch (mechanical or heat allodynia), it can be beneficial. However, when the changes persist, a chronic condition may result (Basbaum *et al.*, 2009). Peripheral sensitization seems to be necessary for the transition from acute pain to the chronic pain states. In most of the acute insults (e.g., surgical incision, sunburn) is the process of nociceptor sensitization reversible and resolved without persistent pain. However, there are many chronic pain states, e.g., neuropathic pain, clearly associated with tissue pathology (e.g., nerve transection or amputation) and irreversible sensitization of nociceptors. Even more mysterious are states of *dysfunctional pain* (such as fibromyalgia, migraine and other, mentioned in CHAPTER 1, p. 5) that occur even in absence of tissue inflammation or other apparent pathology (Gold & Gebhart, 2010).

The causes of *inflammatory pain* (after tissue injury) and *neuropathic pain* are fundamentally different. However, there are many *common mechanisms* responsible for the development of both pain states. Mechanisms involved in both inflammatory and neuropathic pain include (*I.*) altered expression of voltage-gated sodium channels in the DRG, (*II.*) altered expression of cytokines, chemokines and their respective receptors, (*III.*) enhanced glutamate release and receptor functions, (*IV.*) glial and immune cells activation/invasion into the DH of spinal cord and (*V.*) disinhibition (Xu & Yaksh, 2011).

The main difference between inflammatory and neuropathic is that pain after tissue-injury associated injury typically subsides after tissue healing and remission of the inflammation. In contrast, neuropathic pain often persists despite an evident resolution of injury with which the sensation is associated (Xu & Yaksh, 2011). However, in some instances, e.g., after surgery, the inflammatory/injury state may resolve but a component of pain persists—the phenomenon of persistent postoperative pain (Kehlet *et al.*, 2006). Differences also lie in the *involvement of Aβ-fibers* (allodynia, a typical component of the neuropathic pain states reflects the involvement of Aβ-LTMRs), and in *different pharmacology*, which is suitable for treatment of inflammatory or neuropathic pain. While inflammatory pain states are frequently well sensitive and treatable with *nonsteroidal anti-inflammatory drugs* (NSAIDs) or *opiates*, neuropathic pain states not (Xu & Yaksh, 2011). Therefore, it is important to recognize and understand the mechanisms of neuropathic pain to develop better treatment strategies, which may help patients with persistent pain.

4.1. The Role of Non-Neuronal Cells in Pain Processing and Neuroinflammation

Accumulating evidence suggests that non-neuronal cells such as *glial cells*, *immune cells*, *keratinocytes*, *cancer cells*, and *stem cells* play an active role in the modulation of nociceptive transmission and in the pathogenesis of pain (Ji *et al.*, 2016). An overview of released mediators and a scheme of this bilateral communication between non-neuronal cells and nociceptors/spinal nociceptive neurons is summarized in the FIGURE 4.1 (p. 33).
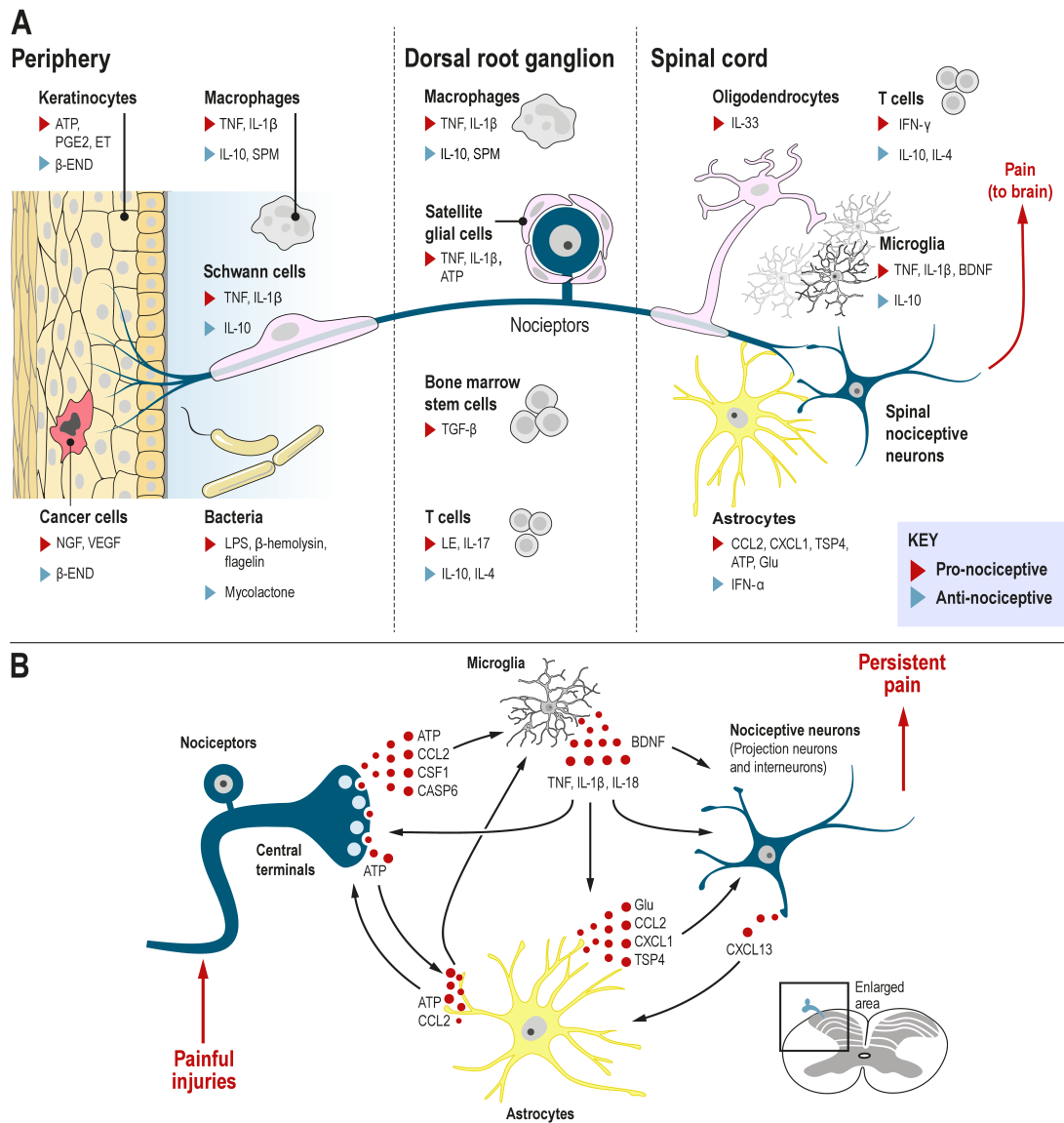
Figure 4.1.: **The role of neuron-glia interaction in the pain plasticity.** (**A**) Interaction of non-neuronal cells with different parts of the pain pathway. Non-neuronal cells produce both pro-nociceptive (the red triangles) and anti-nociceptive (the blue triangles) mediators. These mediators bind to their respective receptors on the nociceptors to modulate its sensitivity and excitability. (**B**) Neuron-glial interaction in the spinal cord level as a mechanism responsible for the central sensitization and amplification of chronic pain. Painful diseases and injuries such as nerve injury, arthritis, cancer and treatment (chemotherapy) cause hyperactivation of nociceptors and release of modulators of glial cells from central endings of nociceptive neurons. These glial modulators lead to the activation of microglia and astrocytes in the spinal cord DH. *Abbreviations:* ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; β-END, β-endorphin; CASP6, caspase 6; CCL2, chemokine (C-C motif) ligand 2; CSF1, colony-stimulating factor-1; CXCL, chemokine (C-X-C motif) ligand (1 or 13); ET, endothelin; Glu, glutamate; IFN, interferon (α/γ); IL, interleukin (IL-1β, -4, -10, -17, -33); LE, leukocyte elastase; LPS, lipopolysaccharide; NGF, nerve growth factor; PGE$_2$, prostaglandin E$_2$; SPMs, specialized pro-resolution mediators (e.g., resolvins, protectins, and maresins, derived from omega-3 unsaturated fatty acids); TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; TSP4, thrombospondin-4; VEGF, vascular endothelial growth factor (Adapted from Ji *et al.*, 2016).

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4.1.1. Glial Cells

Glial cells have been traditionally considered to perform an essentially homeostatic role in the CNS. They are located both in the CNS and PNS. In general, there are several types of glial cells in the CNS, including *astrocytes*, *oligodendrocytes*, *microglia*, and *ependymal cells*; in the PNS, glial cells include *satellite cells* and *Schwann cells*. They offer physical support and protection for neurons, improve synaptic efficacy and maintain an appropriate ionic and physical environment for neuronal activity (Millan, 1999). However, after the injury/insult they may become active and by increased production of inflammatory mediators may critically contribute to *neuroinflammation* (Bradesi, 2010; Ji *et al.*, 2016; Milligan & Watkins, 2009). Spinal cord glial activation[2] and chronic neuroinflammation seems to be a common underlying mechanism of several pain syndromes with widely different etiologies, e.g., atherosclerosis, rheumatoid arthritis, cancer, diabetic neuropathy, chemotherapy-induced neuropathy, and trauma of peripheral nerve or spinal cord (Bradesi, 2010; Milligan & Watkins, 2009).

ASTROCYTES, the most abundant glial cell type in the CNS (40–50 %), are developmentally derived from the *neuroectoderm*. During the embryonic development, the spinal cord contains multiple different classes of astrocytes with different morphology. The individual classes of astrocytes have specific localization and function in the mature intact spinal cord (Miller *et al.*, 1994; Milligan & Watkins, 2009). In the intact tissue, astrocytes perform numerous important functions, such as the formation of the blood-brain barrier (BBB), regulation of extracellular ion concentration and neurotransmitter recycling (Ji et al., 2016). Synaptically released glutamate is from synaptic cleft carried out by two glutamate transporters, mostly by glutamate transporter-1 (GLT-1) and by the glutamate-aspartate transporter (GLAST). These two transporters in naive animals were only observed in astrocytes and not in either microglia or neurons. However, following nerve injury (partial sciatic nerve ligation; PSNL) the total expression of both GLT-1 and GLAST protein decreased and also the cellular location was altered, because both transporters were found in activated microglia following PSNL. Disruption of glutamate recycling causes an elevation extracellular glutamate concentration, which may produce spontaneous pain behavior and increased sensitivity to non-painful stimuli (Xin *et al.*, 2009). It is estimated that single astrocyte can form in rodents 140.000 synapses with 4–6 neuronal somata, and can contact 300–600 neuronal dendrites (Ji *et al.*, 2013). Unlike other glial cells, astrocytes form physically coupled networks mediated by *gap junctions* (connexin-43; Cx43), which allows the exchange of cytosolic contents, including intercellular transmission of ions (Ca^{2+} signaling, K^+ homeostasis). Nerve injury induces increased expression of Cx43 and induce also switch of the function of Cx34 from gap-junction communication to paracrine modulation which will cause an increased release of glutamate and pro-nociceptive modulators, such as ATP and chemokines, through a paracrine mechanism (Chen *et al.*, 2014a). Among the

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[2] GLIAL ACTIVATION is the process of transcriptional and/or translational changes (gene expression), post-translational changes (phosphorylation), morphological changes and the proliferation of glial cells in the PNS and CNS, which is implicated in the development and maintenance of chronic pain (Ji *et al.*, 2014).

nerve injury, there are several others conditions (e.g., spinal cord injury, paw incision, inflammation, arthritis, bone and skin cancer, diabetes, chemotherapy, HIV neuropathy, and chronic opioid treatment), in which astrocytes also become active and lead to the production of many inflammatory mediators (see FIGURE 4.1, p. 33). In comparison with microglia or satellite glial cells, markers of astrocytes are upregulated in most different pain conditions (Ji *et al.*, 2013).

OLIGODENDROCYTES play an important role in the creation of myelin sheath that provides mechanical support and electrical insulation to axons within CNS. In comparison with astrocytes, spinal cord oligodendrocytes are a relatively homogenous population (Miller *et al.*, 1994). The full role of oligodendrocytes in the modulation of nociceptive signaling and pain pathogenesis is largely unknown and the study is still in infancy (Bradesi, 2010). However, recently has been revealed the role of oligodendrocytes in the context of nerve injury-induced neuropathic pain (Zarpelon *et al.*, 2016) and in HIV-associated pain pathogenesis (Shi *et al.*, 2016).

MICROGLIA constitute 5–12 % of all cells heterogeneously distributed throughout the CNS. In contrast to all of the other glial cells, microglia are developmentally derived from *mesodermal cell lineage*[3] commonly with macrophages. Therefore are microglia called as resident macrophages of the spinal cord and brain (Bradesi, 2010; Ji *et al.*, 2016). There is a wide range of conditions (e.g., nerve injury, spinal cord injury, paw incision, ischemia, pathogens etc.), which are considered to be a trigger for switching microglia into an active state (Bradesi, 2010). Even a minor pathological change in the CNS can lead to their rapid activation. However, it has been shown that microglia actively sense their environment and dynamically interact with synapses also under normal physiological conditions in a healthy brain (Ji *et al.*, 2013). The signals that activate microglia include ATP, chemokines CCL2 and CX3CL1 (fractalkine), cytokine CSF1 (colony stimulating factor-1), and proteases (e.g., caspase-6; CASP6). These mediators originate from injured tissue and activated sensory neurons. Simultaneously with nerve injury-induced activation of spinal microglia, expression of the receptors for ATP (purinergic receptors $P2X_4R$, $P2X_7R$, $P2Y_{12}R$) and fractalkine receptor (CX3CR1) is increased. Activation of these receptors typically leads to the expression and subsequent release of several mediators. Pro-nociceptive mediators released by microglia in the spinal cord include $TNF\alpha$ or IL-1β/6/18, PGE_2, and BDNF, see FIGURE 4.1, p. 33 (Grace *et al.*, 2014; Ji *et al.*, 2013; Ji *et al.*, 2016). Some of these mediators, e.g., $TNF\alpha$ or IL-1β/6, enhance excitatory synaptic transmission, but on the other hand, they suppress inhibitory transmission in superficial laminae of the DH (Clark *et al.*, 2015; Kawasaki *et al.*, 2008; Špicarová *et al.*, 2011). Also BDNF via downregulation of K^+/Cl^- co-transporter KCC2 lead to disruption of Cl^- ions homeostasis and consequently to disinhibition in the DH projection neurons (Ferrini *et al.*, 2013).

SCHWANN CELLS together with satellite cells represent the largest population of glial cells in the PNS. Both of them may contribute to pain and usually are activated before central glia.

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[3] More specifically, microglia derived from bone marrow-derived monocytes that migrate during the perinatal development into the CNS (Ji *et al.*, 2016).

After the nerve injury, Schwann cells together with axons and macrophages are involved in the *Wallerian degeneration* that refers to the morphologic and biochemical changes and occurs distal to a site o nerve injury. These changes support the regeneration of the nerve (Conforti *et al.*, 2014). Activated Schwann cells mediate following nerve injury disruption of the BBB via secretion of MMP-9 (matrix metalloproteinase-9), which enhance the recruitment of immune cells (e.g., macrophages) from the vasculature and promotes production and release of proinflammatory mediators, e.g., TNFα, IL-1β, nitric oxide, CCL2, IL-6, and leukemia inhibiting factor (LIF)(Calvo *et al.*, 2012; Shubayev *et al.*, 2006; Tofaris *et al.*, 2002; Zochodne *et al.*, 1999).

Satellite Cell are located in the DRG around the somata of sensory neurons and are directly coupled into the network via gap junctions. After the nerve injury, satellite glial cells become active, proliferate and increase their expression of activation markers such as glial fibrillary acidic protein (GFAP). They also up-regulate the expression of proinflammatory cytokines and their receptors which subsequently leads to an increase in neuronal excitability (Calvo *et al.*, 2012; Ji *et al.*, 2016).

4.1.2. Immune Cells

A diverse form of damage and injury of both PNS and CNS results in substantial recruitment and activation of immune cells. Infiltration of Monocytes and Macrophages into the spinal cord after nerve injury is limited. However, macrophages and also T-Lymphocytes may infiltrate the DRG and surround the cell bodies of injured neurons. Monocytes and macrophages can produce pain through the release of proinflammatory mediators (e.g., TNFα, IL-1β), resulting in increased excitability via modulation of TRPA1, TRPV1, and Na$_v$1.7–1.9 sodium channels. Also, T-lymphocytes are critically involved in the development of neuropathic pain; they produce after infiltration to the DRG pro-nociceptive mediators, such as leukocyte elastase (LE) and interferon γ (IFNγ). In addition, IFNγ via its IFNγ-receptor also mediates activation of spinal microglia, resulting in mechanical allodynia (Calvo *et al.*, 2012; Ji *et al.*, 2016; Robertson *et al.*, 1997; Tsuda *et al.*, 2009).

4.1.3. Other Non-Neuronal Cells

Keratinocytes are the most abundant cell types of epidermis. Keratinocytes are often adjacent to the free nerve endings of nociceptors and other sensory neurons. Their dynamic interactions with sensory nerve endings strongly influence our sense of touch, pain, and itch (Luo *et al.*, 2015). Keratinocytes express, similarly to sensory neurons, thermosensitive transient receptor potential channel 3 and 4 (TRPV3 and TRPV4; Premkumar & Abooj, 2013). Activated keratinocytes may produce various neuroactive paracrine mediators, including ATP, IL-1β, PGE$_2$, endothelin, histamine, NO, or NGF that are known as pro-nociceptive and pain/itch eliciting mediators (Ji *et al.*, 2016; Luo *et al.*, 2015). Keratinocytes can release also various inflammatory

cytokines and chemokines, although they are not immune cells. These mediators can regulate the expression and function of many ion channels in sensory neurons, which may promote hyperexcitability of sensory terminals. It illustrates that not only glial or immune cells may regulate inflammation (Calvo *et al.*, 2012; Luo *et al.*, 2015).

CANCER CELLS may also, among other things, cause cancer pain. It is caused by tumors that metastasize to the bone and infiltrating the nerves. Cancer pain is a complex state, involving inflammatory, neuropathic, ischemic, and compressive mechanisms. Cancer cells may produce and release pro-nociceptive mediators, such as bradykinin, PGE_2, proteases, and endothelins. In addition, cancer cells secrete also growth factors NGF and VEGF. NGF promotes hyperinnervation of nociceptors in cancer tissue and VEGF stimulates the formation of new blood vessels in the cancer tissue. However, VEGF may increase directly nociceptor excitability via VEGF receptor 1 that is expressed in nociceptors (Mantyh, 2013; Selvaraj *et al.*, 2015).

STEM CELLS, e.g., bone marrow stem cells (BMSCs), help to tissue regeneration and they can also effectively regulate inflammation and neuroinflammation. Transplantation of BMSCs into spinal cord suppresses nerve injury-induced glial activation and neuropathic pain by secretion anti-inflammatory cytokine TGF-β1 (Chen *et al.*, 2015). This paracrine modulation of neuroinflammation and pain by BMSCs is different from other stem cell strategies, used for chronic pain treatment (Ji *et al.*, 2016). For example, transplantation of cortical precursors of GABAergic interneurons from embryonic *medial ganglionic eminence* can reverse mechanical allodynia and heat Hyperalgesia produced in a paclitaxel-induced chemotherapy model of neuropathic pain, because these precursor cells differentiate into functional GABAergic interneurons (Braz *et al.*, 2015).

BACTERIA, together with other viral or fungal pathogens, may cause infections commonly associated with inflammation and pain. Bacterial infection may elicit pain indirectly through the activation of immune cells and their inflammatory mediators. However, there are also direct signaling pathways, how bacteria may modulate pain perception directly (Ji *et al.*, 2016). Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria and it is known that LPS may produce pain hypersensitivity by sensitization of TRPV1 receptor in nociceptors. This effect of LPS on TRPV1 is mediated via Toll-like 4 receptors (TLR4; Diogenes *et al.*, 2011; Li *et al.*, 2015a). Among other pro-nociceptive mediators, released by bacteria, belongs pore-forming toxin α-hemolysin and flagellin (ligand of TLR5). In both non-neuronal and neuronal regulation of pain by bacterial infection (and also nerve injury) are critically involved mechanisms through Toll-Like Receptors (TLRs). TLRs are expressed by neurons, glial cells, and immune cells. TLRs are activated by exogenous substances (infection via LPS, flagellin), as well by endogenous ligands, such as high mobility group box 1 protein (HMGB1) or micro/single-stranded RNAs, released after tissue damage (Ji *et al.*, 2016).

4.2. Animal Models of Inflammatory Pain

There are two animal models of inflammation commonly used to study inflammatory mediators, anti-inflammatory drugs and mechanisms involved in the development of inflammatory pain (Fehrenbacher *et al.*, 2012).

The first commonly used model in pain research is Carrageenan-induced paw edema. It is used in rodents for induction of acute inflammation by the subcutaneous injection of 0.5–3 % solution of carrageenan in saline into the plantar surface of the hind paw. Carrageenan induces an acute swelling that becomes maximal 3–5 hours after the injection and it lasts for ∼24 hours (Winter *et al.*, 1962).

The second model used an injection of Complete Freund's adjuvant (CFA) in saline. CFA is composed of an oil emulsion and an immunostimulant (inactivated *mycobacteria tuberculosis*). CFA is used to induce sub-chronic or chronic-like inflammation, e.g. CFA-induced arthritis. CFA produces a more prolonged swelling that becomes maximal at 24 hours and persists for at least 7 days (Iadarola *et al.*, 1988). It has been demonstrated that a single intradermal injection of CFA induces a rheumatoid arthritis-like pathology in the hind paw over a period of 2 weeks (Chillingworth & Donaldson, 2003).

5. The Role of TRPV1 in Signal Transduction and Modulation of Nociception

Transient receptor potential (TRP) *vanilloid type 1* (TRPV1), previously called as vanilloid receptor 1 (VR1) or simply as capsaicin receptor, is one of the six members of vanilloid subfamily (TRPV1–TRPV6) of the TRP channels family. TRPV1 was the first cloned and molecularly characterized nociceptive TRP channel (Caterina *et al.*, 1997).

TRP channels assemble as homo- or hetero-tetramers to form cation-selective channels with diverse modes of activation. Each TRP subunit contains six putative transmembrane segments (S1–S6) with a pore-forming *"reentrant loop"* between S5 and S6 segment (Owsianik *et al.*, 2006).

TRP channels represent a large family of ion channel proteins, which are activated and regulated through strikingly diverse mechanisms, making them suitable candidates for cellular sensors. They respond to stimuli from the environment (such as temperature, pheromones, plant compounds/irritants) and also they have a homeostatic function (maintenance of Ca^{2+} and Mg^{2+} ion equilibrium as well as control of osmolarity and pH; Wu *et al.*, 2010). Over the past two decades, research has revealed that many of TRP channels, e.g., TRPV1–4, TRP melastatin 2, 3, 8 (TRPM2/3/8), TRP ankyrin 1 (TRPA1) and TRP canonical 1, 5, 6 (TRPC1/5/6), are fundamental molecules that detect noxious stimuli and transduce a diverse range of physical and chemical stimuli into action potentials in somatosensory nociceptors (Kádková *et al.*, 2017; Premkumar & Abooj, 2013; Špicarová *et al.*, 2014b; Stucky *et al.*, 2009).

5.1. TRPV1 as an Integrator of Nociceptive Stimuli

TRPV1 may be activated by a wide range of chemical and physical noxious stimuli, and therefore TRPV1 are sometimes called as a polymodal receptor or as a molecular integrator of nociceptive stimuli (Caterina & Julius, 2001; Tominaga *et al.*, 1998). TRPV1 is activated by noxious heat with threshold >43 °C. However, this threshold is reduced in acidic conditions, when moderately low pH < 6.0 cause activation of TRPV1 even at room temperature (Tominaga & Caterina, 2004; Tominaga *et al.*, 1998). Moreover, TRPV1 is activated by a number of (*I.*) *endogenous* and (*II.*) *exogenous*, natural and synthetic compounds.

(*I.*) Endogenous Activators include, e.g., (*a*) lipidic neurotransmitter Anandamide (*N*-arachidonoylethanolamine; AEA), derived from arachidonic acid, which is one of the first observed endogenous TRPV1. AEA is also agonist of metabotropic cannabinoid CB_1 and CB_2 receptors (Zygmunt *et al.*, 1999); (*b*) Eicosanoids and Leukotriene Lipids derived from arachidonic acid[1] by lipoxygenases activity (e.g., 12- and 15-(*S*)-hydroperoxyeicosatetraenoic acid (12- and 15-(*S*)-HPETE), 5- and 15-(*S*)-hydroxyeicosatetraenoic acid (5- and 15-(*S*)-HETE) and leukotriene B4 (LTB4) (Hwang *et al.*, 2000); (*c*) the oxidized Linoleic Acid Metabolites, such as 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE) and their metabolites, 9- and 13-oxoODE, which are formed in mouse and rate skin biopsies after exposure to noxious heat (Patwardhan *et al.*, 2010); (*d*) TRPV1 may also be activated by Fatty Amides, such as *N*-oleoyl-dopamine (OLDA, derived from carboxy group of oleic acid and dopamine; Chu *et al.*, 2003); and *N*-arachidnoyl-dopamine (NADA, derived from a dopamine and an arachidonic acid, also agonist of CB_1 receptor; Huang *et al.*, 2002).

(*II.*) Exogenous Activators include, e.g., Capsaicin, the active ingredient in hot chili peppers (Caterina *et al.*, 1997); Resiniferatoxin, the ultra-potent capsaicin analog, agonist from the plant *Euphorbia resinifera* (Szolcsanyi *et al.*, 1990); Eugenol and Guaiacol obtained from the oil of cloves—*Eugenia caryophyllata*; Piperine from black pepper (*Piper nigrum*); Zingerone from ginger (*Zingiber officinale*); Scutigeral from fungi *Albatrelus ovinus*; Allicin, the pungent substance of garlic, onion and other plants from the *Allium* genus (Salazar *et al.*, 2008); and synthetic vanilloids such as Olvanil, Nuvanil (Szallasi & Blumberg, 1999) and many other synthetic agonists developed by pharmaceutical companies as a potential therapeutics.

There are also a number of substances that do not activate TRPV1 directly but modulate, e.g., by phosphorylation, its properties through multiple signaling cascades and second messengers. Thus, for example, the inflammatory mediators lead to sensitization of the TRPV1 and to the development of behavioral hypersensitivity, allodynia, and hyperalgesia (see next Section 5.3; p. 42).

TRPV1 is a non-selective cation channel. Its activation leads to the membrane depolarization to ~ 0 mV and to a robust increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). TRPV1 does not discriminate among monovalent cations, but exhibits a notable preference for divalent cations ($Ca^{2+} > Mg^{2+} > Na^+ \approx K^+ \approx Cs^+$). The relative permeability of TRPV1 to Ca^{2+} ions is ten-times higher than to Na^+ ions; in case of Mg^{2+} ions, permeability is five-times higher compared to Na^+ (Caterina *et al.*, 1997; Oh *et al.*, 1996). Due to high calcium permeability, TRPV1 may induce a robust increase of $[Ca^{2+}]_i$ and neurosecretion independently on action potential. It has been reported that a brief stimulation of presynaptically located TRPV1 with agonist capsaicin or endogenous agonist NADA induces a robust and prolonged

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[1] Arachidonic acid and its products are produced and released by DRG neurons, for example, when exposed to bradykinin (BK). BK through its G-protein coupled receptor leads to activation of phospholipase A_2, which by the cleaving of membrane lipids in DRG cells produce arachidonic acid—a lipoxygenase substrate (Thayer *et al.*, 1988).

elevation of presynaptic $[Ca^{2+}]_i$ and subsequent enhancement of neurotransmitter release at sensory synapses. This TRPV1-mediated response was induced also in medium, where action potential propagation and voltage-gated calcium channels (VGCCs) were completely blocked by the low-Na^+/TTX, and Cd^{2+} ions-containing solution (Medvedeva *et al.*, 2008).

5.2. Distribution of the TRPV1 in an Organism

The TRPV1 is expressed at low levels throughout a wide range of CNS and peripheral tissues. The complementary DNA of human TRPV1 orthologue show significant homology to the rat TRPV1—86 %, and 92 % similarity at the amino acid level. Both rat and human TRPV1 were found to be most highly expressed in DRG/trigeminal ganglia. However, pronouncedly lower TRPV1 expression has been also found in other tissues, e.g., in the pancreas, different areas of the brain (such as cerebellum, hippocampus and frontal cortex), testis, kidney, liver, spleen, uterus, spinal cord, lung and other tissue (Hayes *et al.*, 2000).

Within the DRG/trigeminal ganglia of rats, TRPV1 expression/immunoreactivity is restricted to small- and medium-sized neurons with Aδ and C-fibers (Caterina *et al.*, 1997; Guo *et al.*, 1999). TRPV1 is transported into both central and peripheral processes of these primary afferent neurons, and therefore, high immunoreactivity has been found in superficial laminae I–II of the DH, and the TRPV1-positive small-diameter nerve fibers have been found also in the skin and cornea in the rat (Guo *et al.*, 1999). A high density of TRPV1-positive central terminals in the DH of the spinal cord has been also revealed by autoradiography method, by radioactively labeled ultra-potent TRPV1 agonist [^{3}H]resiniferatoxin in human spinal cord specimens removed post-mortem (Szallasi *et al.*, 1994). Distribution of TRPV1 throughout different spinal cord segments is not uniform. It showed a similar density in the cervical and thoracic segments, while, it was twice as high in the lumbar segment (Szallasi *et al.*, 1995). More detailed analysis of the lumbar segment revealed that the density or TRPV1-positive fibers and boutons in the DH increases progressively in a rostrocaudal direction, from spinal segments L4 to L6 (Hwang & Valtschanoff, 2003). Dorsal rhizotomy (i.e., transection of dorsal roots) or systemic treatment with toxic dose of capsaicin abolishes TRPV1 staining in the DH of the spinal cord, which suggests that the majority of TRPV1 is located presynaptically in the fibers of primary afferent DRG neurons (Szallasi *et al.*, 1995; Valtschanoff *et al.*, 2001).

It has been also showed that TRPV1 expression is different in the DRG and in the DH at different postnatal ages in the mouse. During early postnatal development P1–P8, TRPV1 has been found in a wide range of DRG neurons ($\sim$60 %). Then, the number of TRPV1 positive DRG neurons dramatically decreased to $\sim$30 %, and become restricted to a specific subset of the peptidergic neurons on P15–P22 and stay constant on this level in the adult mouse. Similarly, the TRPV1 staining is dramatically decreased within the DH and become progressively restricted to the outer DH laminae in adult animals (Cavanaugh *et al.*, 2011).

5.3. TRPV1 Modulation and its Role in the Pathological Pain States

The role of TRPV1 in periphery and mechanisms of its modulation in the periphery are relatively well known. Peripheral TRPV1 may be activated directly by several chemical compounds and physical stimuli, mentioned above. However, the function of spinal, and central TRPV1 receptors in general, as well as mechanisms and consequences of their modulation in the nociceptive transmission and chronic/neuropathic pain are not fully understood. Unlike the peripheral receptors, the central TRPV1, expressed in the DRG and spinal cord can hardly respond to high temperatures or acid/low pH in the environment. Nevertheless, a wide range of modulators may sensitize both peripheral and central TRPV1. These modulators, which may be found in elevated concentrations especially in pathological conditions, act primarily on other receptors, but they may via different signaling pathways and second messengers modulate the function and expression of TRPV1. For example, inflammatory mediators, such as bradykinin, sensitizes TRPV1 by phosphorylation and reduce the activation temperature threshold up to body temperature (Sugiura *et al.*, 2002). It leads to sensitization of peripheral nociceptive endings to heat, but more interestingly, it may activate also central/spinal TRPV1 by body temperature.

TRPV1-mediated responses are robustly potentiated by many proinflammatory agents and trophic factors, including, e.g. *bradykinin* (BK), *glutamate, histamine, serotonin, tripsine, NGF, GDNF, insulin,* and *insulin-like growth factor 1* (IGF-1) (Premkumar & Abooj, 2013). Spinal TRPV1 may also be modulated/sensitized by proinflammatory cytokines and chemokines, such as CCL2 (Špicarová *et al.*, 2014a), TNFα (Špicarová & Paleček, 2010). Many other mediators and second messengers are also implicated in the modulation of TRPV1, such as ATP (Lishko *et al.*, 2007), *phosphatidylinositol 4,5-bisphosphate* (PIP$_2$) (Lukacs *et al.*, 2007) or *prostaglandins* (Moriyama *et al.*, 2005; Zhang *et al.*, 2008).

Many of these mediators mediate sensitization of TRPV1 receptors and positively regulate its expression via different kinases—including *PKC, PKA, CaMKII,* and *PI3K*. It may be responsible for the development of hypersensitivity and pathologic pain states.

Protein Kinase C (PKC) mediated phosphorylation of TRPV1 play a pivotal role in the pathology of the pain (Premkumar & Abooj, 2013). Activation of PKC by activator phorbol ester was showed to potentiate TRPV1 mediated responses and to depolarize sensory neurons (Dray *et al.*, 1988; Dray *et al.*, 1992; Premkumar *et al.*, 2004). PKC-mediated phosphorylation greatly increased TRPV1 sensitivity also to endogenous substances NADA (Premkumar *et al.*, 2004) and *N*-oleoylethanolamide (OEA) that normally did not activate TRPV1 (Ahern, 2003), or even it behaves as an antagonist (Almasi *et al.*, 2008). PKC pathway is involved in the modulatory action of proinflammatory chemokines, such as CCL2. It has been shown that CCL2 via PKC regulates by phosphorylation function of TRPV1 and Na$_v$1.8 channels in DRG neurons (Špicarová *et al.*, 2014a; Zhao *et al.*, 2014).

Protein Kinase A (PKA; known also as cyclic adenosine monophosphate (cAMP)-dependent protein kinase) plays important role in TRPV1 modulation plays as well, e.g., in the

development of prostaglandin E$_2$-induced inflammatory hyperalgesia (Jeske *et al.*, 2008).

For both PKC- and PKA-mediated phosphorylation of TRPV1 is necessary scaffolding protein A-kinase anchoring protein (AKAP)[2]. Administration of AKAP150/79 antagonist significantly reduced PGE$_2$/PKA-induced sensitization to thermal stimuli *in vivo* and significantly reduced TRPV1 phosphorylation in vitro in the culture of trigeminal neurons (Jeske *et al.*, 2008). AKAP150/79 also promotes BK/PKC-mediated TRPV1 phosphorylation (Zhang *et al.*, 2008). Among PKA and PKC, AKAP150/79 also bind Ca^{2+}/calmodulin-dependent phosphatase calcineurin that plays an important role in the desensitization of TRPV1 (see below SECTION 5.4).

Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II (CaMKII) plays an important role in Ca^{2+}-dependent modulation of TRPV1 activity. CaMKII-mediated phosphorylation seems to be necessary, e.g., for TRPV1 responsiveness to capsaicin (Jung *et al.*, 2004). The dynamic balance between Ca^{2+}-dependent phosphorylation or dephosphorylation mediated by CaMKII, resp. by phosphatase calcineurin plays a fundamental role in the control of TRPV1 responsiveness and desensitization (Nagy *et al.*, 2014).

PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) has been found to play important role in the development of inflammatory and neuropathic pain, among other through TRPV1 modulation (Kao *et al.*, 2012; Pezet *et al.*, 2008; Zhuang *et al.*, 2004). For example, PI3K has been shown effective to produce strong sensitization of TRPV1-mediated capsaicin responses upon NGF stimulation (Stein *et al.*, 2006; Zhang *et al.*, 2005). A similar mechanism of PI3K-mediated sensitization of TRPV1 responses to capsaicin was recently reported by our group in a model of paclitaxel-induced peripheral neuropathy (Adámek *et al.*, 2019). PI3K binds to TRPV1 directly through its catalytic subunit p85 and increased responsiveness to capsaicin may be a consequence of increased TRPV1 trafficking to the plasmatic membrane (Stein *et al.*, 2006; Zhu & Oxford, 2007).

It is important to keep in mind that functional TRPV1 channel does not work in plasma membrane alone, but rather, it is integrated into a "TRANSDUCTOME". It is a *macromolecular complex* including scaffolding proteins, such as AKAP, and downstream signaling molecules, including PKC, phospholipase C, calmodulin, PI3K (Nagy *et al.*, 2014; Stein *et al.*, 2006), and also vesicular proteins synaptotagmin IX and snapin, which may via PKC signaling promote the *SNARE-dependent exocytosis*[3] of TRPV1 from cytosolic vesicles to the cell surface (Morenilla-Palao *et al.*, 2004). More recently, direct interaction between TRPV1 and TLR4 was also reported. For this association is required TIR domain of TLR4. This interaction was found to enhance TRPV1 activity by blocking activation-induced TRPV1 desensitization (Min *et al.*, 2018).

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[2] The AKAP family of scaffolding protein were originally named for their ability to bind PKA and target it to an appropriate substrate but now it is known that AKAP may assemble with a wide range of kinases and phosphatases into signaling complexes. AKAP79 is the human homolog, while AKAP150 is the rodent homolog. Both AKAP79 and 150 seems to be similar in function (Zhang *et al.*, 2008).

[3] The SNARE (Soluble NSF (*N*-ethylmaleimide-sensitive factor) Attachment REceptor)-fusion protein complex represent the main apparat for fusion of synaptic vesicles with the plasma membrane (Karmakar *et al.*, 2019).

5.4. Desensitization and Tachyphylaxis of TRPV1

Prolonged activation of TRPV1, e.g., by vanilloids, heat or protons, induces ACUTE DESENSI-TIZATION of the TRPV1 receptor and subsequently desensitizes nociceptive nerve fibers. This process of acute desensitization contributes to the analgesic effect of topically used vanilloids (Nagy *et al.*, 2014; Touška *et al.*, 2011). Depending on the duration of TRPV1 receptor activation and the extracellular concentration of Ca^{2+}, calcium influx via TRPV1 activates signaling that desensitizes TRPV1 itself. This mechanism represents a feedback mechanism, which protects nociceptive neurons from toxic Ca^{2+} overload (Touška *et al.*, 2011), because it is known that prolonged activation of TRPV1, e.g., by capsaicin, induces cell death (Lawson, 1987).

The other term, TACHYPHYLAXIS, describes the phenomenon when repeated activation of the receptor, e.g., by capsaicin exposure, induces a reduction in the TRPV1-mediated responses (Koplas *et al.*, 1997; Touška *et al.*, 2011). It was demonstrated that repeated application leads to a decrease in the release of glutamate-evoked by the second application of capsaicin in the dorsal horn of the spinal cord (Ueda *et al.*, 1993).

However, it is not yet clear if tachyphylaxis is induced by similar mechanisms as acute desensitization, but both of these phenomenon share at least some molecular processes (Nagy *et al.*, 2014).

Both acute desensitization and tachyphylaxis are largely Ca^{2+}-dependent processes (Koplas *et al.*, 1997; Piper *et al.*, 1999).

While Ca^{2+}-dependent CaMKII-mediated phosphorylation of TRPV1 is fundamental for its responsiveness to capsaicin, activation of Ca^{2+}/calmodulin dependent protein phosphatase CALCINEURIN and dephosphorylation of TRPV1 has been reported necessary to enhance desensitization and tachyphylaxis to capsaicin (Docherty *et al.*, 1996; Zhang *et al.*, 2011). On the other hand, phosphorylation of TRPV1 by protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin dependent kinase II (CaMKII) have been considered to reverse desensitization/or to produce sensitization (Touška *et al.*, 2011).

In addition, CALMODULIN (CaM) itself plays an important role in the TRPV1 desensitization. TRPV1 has four binding sites for CaM. Ca^{2+}/CaM complex reduces responses of TRPV1 to its activators and produce desensitization and/or tachyphylaxis. This effect may be prevented by ATP binding, which prevents desensitization of TRPV1 (Lishko *et al.*, 2007).

Tachyphylaxis of TRPV1-mediated responses on the cellular level may be strongly regulated also by PI3K. It was demonstrated repeatedly that PI3K regulates TRPV1 trafficking to the plasma membrane. Following PI3K activation, the extent of tachyphylaxis to repeated capsaicin application is reduced and moreover, it may produce sensitization of TRPV1-mediated capsaicin responses (Stein *et al.*, 2006; Zhang *et al.*, 2005).

Since desensitization and tachyphylaxis of TRPV1 receptors contribute to the analgesic effect of TRPV1 activators, it is important and therapeutically relevant to study these mechanisms. Pharmacological modulation of TRPV1 receptors with the aim to induce the desensitized state of TRPV1 may potentially help to alleviate pain in different pain states.

5.5. TRPV1 and Endovanilloid/Endocannabinoid System

Increasing body of evidence suggest that functional interaction between endovanilloid and endocannabinoid system, respectively interaction between TRPV1 and CB receptors play remarkable role, not only in the modulation of nociceptive of synaptic transmission in the dorsal horn (Guindon *et al.*, 2013), but also in modulation of anxiety (Faraji *et al.*, 2017) and vision (Ryskamp *et al.*, 2014).

The terms endovanilloids (eV) and/or endocannabinoids (eCB) refer to the lipidic molecules derived from arachidonic acid. Some of these substance act as agonists for both TRPV1 and CB receptors. For clarity, the abbreviation *eV/eCBs* will be used for both terms in the following text. eV/eCBs mediate various forms of synaptic plasticity at excitatory and inhibitory synapses in the brain (Edwards *et al.*, 2012).

One of the first observed eV/eCBs is lipidic neurotransmitter ANANDAMIDE (AEA). It was already mentioned that AEA is an endogenous agonist of both TRPV1 and metabotropic $G_{i/o}$ protein-coupled cannabinoid CB_1 and CB_2 receptors (Zygmunt *et al.*, 1999). Another well-known lipidic neurotransmitter able to activate both TRPV1 and CB receptors is 2-ARACHIDONOYL-GLYCEROL (2-AG; Zygmunt *et al.*, 2013), then also *N*-ARACHIDONOYL-DOPAMINE (NADA; Huang *et al.*, 2002) and weak CB_1 agonist is also *N*-OLEOYL-DOPAMINE (OLDA; Chu *et al.*, 2003).

Importantly, the main molecule of eV/eCB system—AEA may be synthesized and/or metabolized by sub-population of DRG neurons, as well as spinal cord cells, such as microglia (Carrier *et al.*, 2004; Varga *et al.*, 2014; Vellani *et al.*, 2008).

N-acylphosphatidylethanolamine (NAPE), which is synthesized from membrane phospholipids by *N*-acetyltransferase (NAT), represents the main precursor molecule for AEA synthesis through different enzymatic pathways. AEA is from NAPE synthesized in both intracellular Ca^{2+}-dependent and Ca^{2+}-independent manner (Varga *et al.*, 2014; Vellani *et al.*, 2008).

Key enzymes responsible for the synthesis of AEA and 2-AG in a *Ca^{2+}-dependent* manner include *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD) and *sn*-1-specific diacylglycerol lipase (DAGL). In sensory neurons, the important role in AEA synthesis play as well *Ca^{2+}-independent* enzymatic pathways, including (*I.*) α/β-hydrolase 4 (ABHd4); (*II.*) glycerophosphodiester phosphodiesterase 1 (GDE1); (*III.*) protein tyrosine phosphatase, nonreceptor type 22 (PTPn22); and (*IV.*) inositol 5'-phosphatase (Inpp5). The degradation of AEA and 2-AG is controlled by enzymes fatty acid amide hydrolase (FAAH) and by the monoacylglycerol lipase (MAGL), respectively (Fezza *et al.*, 2014; Varga *et al.*, 2014).

Under the physiological conditions, eCB and cannabinoids, in general, attenuates nociceptive signaling and have an analgesic effect. Therefore, eCB/cannabinoids are considered as therapeutics to alleviate acute and chronic pain (Manzanares *et al.*, 2006; Rahn & Hohmann, 2009). On the other hand, activation of TRPV1 receptors by eV/eCBs is primarily pronociceptive and proinflammatory, because TRPV1 activation may also support inflammation via the release of neuropeptides. Despite this fact, TRPV1 modulation by eV/eCBs may be explicitly

important in pain management and analgesia. It was recently reported that eV/eCBs, concretely palmitoylethanolamide (PAE)—an endogenous congener of AEA, could slightly enhance TRPV1 to 2-AG-induced activation, but PEA produces significant 2-AG-induced desensitization of the TRPV1 as well to more potent agonist, such as capsaicin (Petrosino *et al.*, 2016). This finding suggests a possibility that eCB may via TRPV1 desensitization consequently attenuate the pronociceptive component of eCB-induced TRPV1-signaling, including reduction of TRPV1-mediated release of proinflammatory mediators.

It is also important to mention, that at least in the higher brain areas—e.g., in hippocampus CA1 interneurons, the eV/eCBs-mediated depression at excitatory synapses may be induced by activation of metabotropic glutamate receptor mGlur5 in CB_1/TRPV1 independent manner (Edwards *et al.*, 2012).

Finally, eCBs may modulate nociceptive transmission in the dorsal horn of the spinal cord as well via affecting of inhibitory transmission. Despite that, eCBs play mainly suppressive role in pain signaling, it has been reported that eCBs may promote nociception via activation of CB_1 receptor located on DH IN–INs, which diminishes the inhibitory control and thereby endocannabinoids facilitate nociception (Pernia-Andrade *et al.*, 2009).

The effect of eV/eCBs in the regulation of nociceptive synaptic transmission remain poorly understood. Therefore, part of my experimental work was focused on the study how the administration of 20:4-NAPE, an AEA substrate, affect nociceptive synaptic transmission in the spinal cord and what is the role of TRPV1 and CB_1 receptors in this process under normal/physiological and inflammatory conditions (Nerandžič *et al.*, 2017).

6. Chemotherapy-Induced Peripheral Neuropathy & Neuropathic Pain

Fundamentally, *Chemotherapy-Induced Peripheral Neuropathy* (CIPN) is often accompanied by *neuropathic pain*, because the primary cause of CIPN is an abnormal function and damage of the nervous system, especially PNS. On the other hand, there are several common *neuroinflammatory mechanisms* that CIPN shares with *inflammatory pain* (Xu & Yaksh, 2011).

In general, pain is a common symptom in cancer patients, affecting ∼30–50 % of patients undergoing active anti-cancer therapy of solid tumor and ∼70–90 % of patients with advanced diseases (Portenoy & Lesage, 1999). Since the 1970s, there is a 3-fold increase in the number of cancer survivors worldwide. As a consequence of advances in cancer diagnostics and treatment, there is now a significant number of cancer survivors suffering because of neurotoxicity of chemotherapy, which has a significant impact on quality of their life following cancer treatment (Park *et al.*, 2013).

CIPN is a frequent side effect of the commonly used anti-cancer drugs (Boyette-Davis *et al.*, 2015). Chronic painful CIPN represents only one type of cancer pain. In general, cancer-related pain may be caused directly by (*I.*) tumor infiltration or compression within nervous system; (*II.*) immunoreactive and pronociceptive substances released from tumors; (*III.*) as a side effect of anti-cancer treatment—including chemotherapy, radiation, or surgery (Fallon, 2013; Wang & Wang, 2003).

CIPN is characterized by Paresthesia, Dysesthesia[1] and often by Neuropathic Pain, primarily in limb extremities—in hands and feet. Patients most often report sensory symptoms of numbness and tingling, followed by symptoms described as burning, shooting, stabbing and throbbing (Boyette-Davis *et al.*, 2015).

Severe acute CIPN symptoms may require chemotherapy dose reduction or cessation that may be linked with poorer survival rates. A systematic meta-analysis revealed that the prevalence of CIPN was ∼68 % when measured in the first month after chemotherapy, ∼60 % at 3 months and ∼30 % at 6 months or more (Seretny *et al.*, 2014). There is no effective CIPN prevention strategy; there is only limited evidence of effective drugs for the treatment of established chronic CIPN (Flatters *et al.*, 2017; Seretny *et al.*, 2014).

Several widely used antineoplastic drugs including *paclitaxel, docetaxel, oxaliplatin, cisplatin, vincristine, bortezomib,* and *thalidomide* caused CIPN (Cavaletti, 2014; Park *et al.*, 2013).

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[1] Paresthesia is an abnormal dermal sensation (spontaneous or evoked) that is not unpleasant; In contrast, Dysesthesia is an unpleasant abnormal sensation (spontaneous or evoked; Merskey & Bogduk, 1994).

Table 6.1.: **Summary of commonly used chemotherapeutic drugs and the most frequent symptoms and signs associated with their administration** (Adapted from Cavaletti 2014; Park 2013).

Type	Class	Sensory neuropathy	Motor neuropathy	Autonomic neuropathy
Paclitaxel	Taxane/ Antitubulins	Predominantly sensory neuropathy (upper and/or lower limb impairment/loss of all sensory modalities); neuropathic pain/*paresthesia* (abnormal dermal sensation is frequent)	*Myalgia* (muscle pain; frequently at higher doses) and *myopathy* (distal, symmetric weakness in lower limbs is mild)	Rare
Docetaxel	Taxane/ Antitubulins	Predominantly sensory neuropathy (upper and/or lower limb impairment/loss of all sensory modalities); neuropathic pain/*paresthesia* (abnormal dermal sensation is frequent)	At higher doses *myalgia* and *myopathy* (similar to paclitaxel)	Rare
Oxaliplati	Platinum drug	Acute sensory symptoms (cold-induced *paresthesia* in mouth, throat and limb extremities) and chronic sensory neuropathy	Acute cramps/muscle spasm in throat muscle and fasciculations	Rare
Cisplatin	Platinum drug	Predominantly sensory neuropathy (impairment/loss of all sensory modalities, extreme sensitivity to cold); neuropathic pain is rare	Rare (an influencing of large myelinated fibers may affect gait imbalance)	Rare
Vincristine	Vinca alkaloid	Sensory neuropathy (upper and/or lower limb impairment/loss of all sensory modalities); *paresthesia*/neuropathic pain is frequent at limb extremities	Muscle cramps and mild distal weakness	Yes, and it may be severe (e.g., orthostatic/postural hypotension, constipation, paralytic ileus)
Bortezomib	Proteasome inhibitor	Mild to moderate sensory neuropathy; myelinated and unmyelinated small-fibers are affected leading to severe neuropathic pain	Rare (mild distal weakness in lower limbs)	Rare
Thalidomide	Immuno-modulator/ anti-angiogenic agent	Sensory neuropathy (impairment/loss of all sensory modalities); frequently neuropathic pain at limb extremities	Rare (mild distal weakness and cramps)	Rare

The frequency of symptoms in sensory, motor and autonomic neuropathy categories for each drug is summarized in TABLE 6.1 (p. 48). Different chemotherapies affect distinct components of the nervous system—from the level of the DRG to the distal axonal endings in the periphery. Putative targets for CIPN toxicity and distribution of symptoms is shown in FIGURE 6.1 (p. 50). A prominent target for neurotoxicity represents the DRG (Park *et al.*, 2013) because it is less protected by the BBB, in comparison with CNS, and therefore are DRG neurons more vulnerable to neurotoxic damage (Allen & Kiernan, 1994). That probably explains the predominance of peripheral mechanisms involved in the development of CIPN.

Another important mechanism of neurotoxicity is based on the disruption of microtubule dynamics that impair axonal transport processes including energy and material delivery. Other alterations associated with the CIPN include following changes: (*I.*) alterations in expression/activity of ion channels; (*II.*) alterations of neurotransmission; (*III.*) mitochondrial dysfunction and oxidative stress; (*IV.*) activation of glial cells and engagement of immune cells (resulting in increased production of neuroinflammatory chemokines and cytokines); (*V.*) loss of intraepidermal nerve fibers and Meissner's corpuscle; (*VI.*) Wallerian degeneration; and (*VII.*) damage of the peripheral vasculature, leading to axonal degeneration (Boyette-Davis *et al.*, 2015; Flatters *et al.*, 2017; Park *et al.*, 2013; Siau *et al.*, 2006).

Many animal models have been designed to study CIPN. Chemotherapeutic drugs produce neuropathy also in animals which may be used to study causes, prevention, and treatment of their neurotoxicity (Wang & Wang, 2003).

In our experiments, we are focused on *paclitaxel-induced acute pain* and *chronic painful neuropathy*, and hence, especially paclitaxel-related syndromes and mechanisms will be discussed in more detail in the next section.

6.1. Paclitaxel-Induced Peripheral Neuropathy and Neuropathic Pain

Paclitaxel (PAC; also known as Taxol) is a natural product with an anti-tumor activity that was originally obtained from the bark of the Pacific yew, *Taxus Brevifolia* (Wani *et al.*, 1971). The anti-neoplastic effect of PAC is based primarily on the ability to bind to the cytoskeletal protein tubulin (Arbuck *et al.*, 1993). PAC binds to β-tubulin components inside the microtubules scaffold and stabilizes GDP-bound of $\alpha\beta$-tubulin heterodimers, which produce over-polymerization and inhibition of depolymerization at the minus end of the microtubule. Interference of PAC with normal microtubule dynamics is linked to the disruption of the cell cycle (mitosis) and axonal transport (Nicolini *et al.*, 2015; Park *et al.*, 2013). However, the modulation of apoptotic processes via activation of MAPKs has also been documented (MacKeigan *et al.*, 2000; McDaid & Horwitz, 2001). Taxanes, including PAC, also display immunomodulatory effects (Fitzpatrick & Wheeler, 2003).
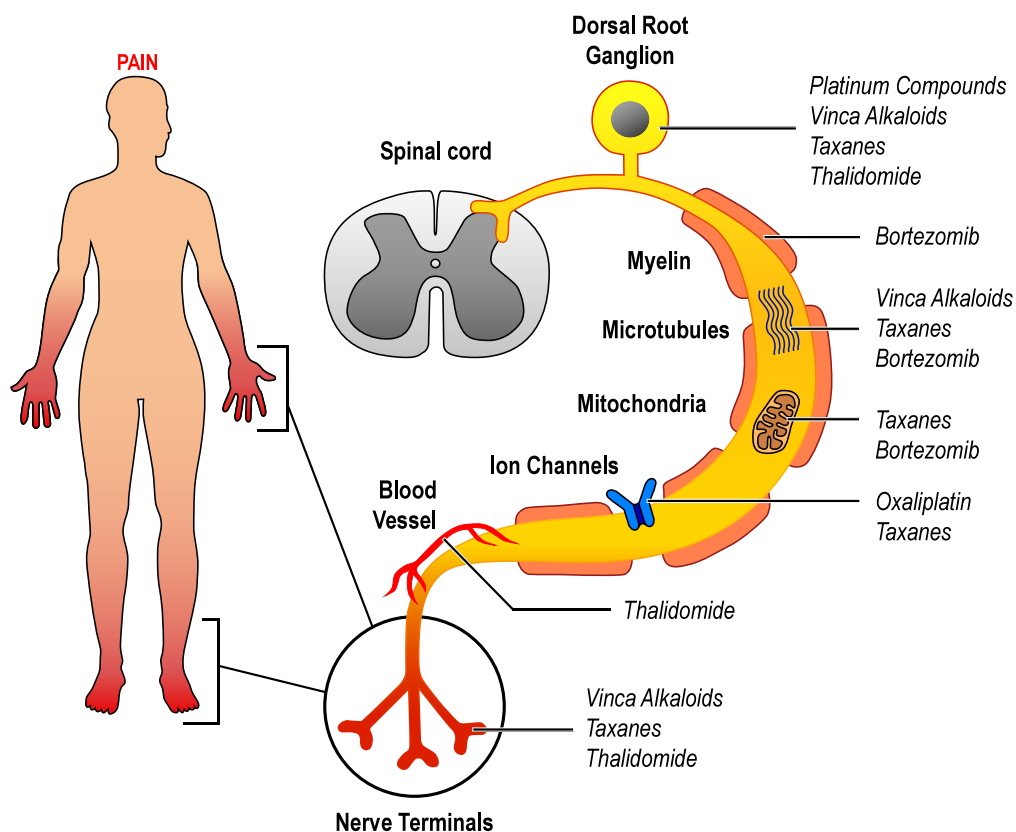
Figure 6.1.: **Scheme of the typical *"Glove-and-Stocking"* distribution of chemotherapy-induced peripheral neuropathy (CIPN) symptoms.** Putative targets for CIPN toxicity in the PNS are shown. Chemotherapeutics may affect PNS from DRG to axonal components (such as microtubules, myelin, ion channels, mitochondria, peripheral vascularization) and distal nerve terminals in the periphery (Adapted from Park *et al.*, 2013).

Although, PAC is a frontline chemotherapeutic agent widely used in clinical practice for the treatment of solid tumors such as breast, ovarian, prostate and lung cancer, there are several clinical problems associated with PAC therapy.

The first commonly reported debilitating side effect of PAC therapy, which significantly impairs the patient's quality of life, is the development of CHRONIC PAINFUL PERIPHERAL NEUROPATHY—CIPN, often accompanied by *chronic neuropathic pain* (Park *et al.*, 2013). In addition to CIPN and chronic neuropathic pain, PAC is also associated with *acute pain syndrome* (P-APS; Reeves *et al.*, 2012; Yan *et al.*, 2015b). Both chronic painful CIPN and P-APS are often resistant to standard analgesic treatments. Despite the widespread use of PAC in clinical practice, mechanisms of both chronic CIPN and P-APS remain insufficiently understood. A large part of our experiments was devoted to a study of the mechanisms of PAC-induced CIPN, in particular with regard to neuropathic pain. Therefore, it will be discussed in more detail in the next SECTION 6.2.

Another obstacle to successful therapy may be a development of CHEMORESISTANCE to PAC (Kim *et al.*, 2007). Chemoresistance is probably mediated via activation of anti-apoptotic

pathways, including activation of MAPK/ERK pathway[2] (Xia *et al.*, 1995) and PI3K/Akt/NFκB pathway[3] (Mabuchi *et al.*, 2004). It has been shown that PAC promotes breast cancer metastasis in a Toll-like receptor 4 (TLR4)-dependent manner (Volk-Draper *et al.*, 2014) and that the inhibition of PI3K signaling synergistically increase the efficacy of PAC-induced apoptosis in many cancer cell lines (Kawaguchi *et al.*, 2007; Kim *et al.*, 2007; MacKeigan *et al.*, 2002).

6.2. Alterations Associated with Paclitaxel-Induced Peripheral Neuropathy and Neuropathic Pain

PAC-induced peripheral neuropathy (PIPN) often becomes a chronic painful condition accompanied by *neuropathic pain*. PIPN represents the serious dose-limiting factor of PAC anti-cancer therapy (Boyette-Davis *et al.*, 2018). The development of PIPN and neuropathic pain is a consequence of numerous changes both on the cellular and molecular level.

Paclitaxel has only limited ability to penetrate into the CNS. It was demonstrated in an *in vivo* rat model that DRG represents the main site of PAC accumulation (Cavaletti *et al.*, 2000; Xiao *et al.*, 2011) and *in vitro* experiments have revealed PAC toxicity in DRG cultures (Scuteri *et al.*, 2006). In fact, DRGs are vascularised by fenestrated endothelial cells lacking tight junctions. It means that DRGs are not protected by a barrier comparable to the BBB; hence, DRGs are exposed to different classes of low and high molecular weight drugs (Cavaletti *et al.*, 2000). These facts explain why PIPN is predominantly reported as a pathology of peripheral sensory neurons (Nicolini *et al.*, 2015). However, it has been reported repeatedly in rodents and also in humans that low concentration of PAC also may penetrate to the cerebral spinal fluid, rodent brain and spinal cord (Cavaletti *et al.*, 2000; Fellner *et al.*, 2002; Gelderblom *et al.*, 2003; Kemper *et al.*, 2003; Yan *et al.*, 2015b). Therefore, PAC may directly produce changes in the PNS and it seems that in low concentration may PAC probably directly modulate the function of CNS, including modulation of spinal nociceptive processing. Eventually, changes in the CNS may also be a secondary consequence of peripheral changes induced primarily by PAC.

The cumulative effect all of these PAC-induced changes discussed in more detail below may cause loss of intraepidermal innervations and loss or damage of Meissner's corpuscles in the rodent model of PAC-induced CIPN. Activation of cutaneous Langerhans cells following PAC treatment also suggests possible neuro-immune interactions that might also have a role in PIPN (Boyette-Davis *et al.*, 2011; Siau *et al.*, 2006). The distribution of these changes correlates to the areas where the symptoms are worst—in hands and feet, i.e., *"Glove-and-Stocking"* distribution, see Figure 6.1, p. 50 (Boyette-Davis *et al.*, 2011).

A growing body of evidence shows that TLR4 play important role in the development of PIPN and PAC-induced neuropathic pain (Li *et al.*, 2015a; Li *et al.*, 2015b; Wu *et al.*, 2015;

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[2] Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinases pathway

[3] Phosphatidylinositol 3-kinase/Akt kinase (protein kinase B)/Nuclear Factor-κB pathway

Yan *et al.*, 2015b). This issue will be discussed separately in more detail in Chapter 7 (p. 55), resp. in Section 7.1 (p. 56).

6.2.1. Paclitaxel and Oxidative Stress

Several studies report damage of neuronal mitochondria as a result of chemotherapy with PAC, which is manifested by the increased prevalence of abnormal swollen and vacuolated mitochondria, in the myelinated and unmyelinated sensory axons, but not in the motor axons (Flatters & Bennett, 2006; Xiao *et al.*, 2011).

Damaged mitochondria are known to produce *reactive oxygen species* (ROS), which can, in turn, worsen mitochondrial function. Hence, several groups have explored the contributory role of ROS *in vivo* to PAC-induced pain behavior. Administration of free radical scavengers was shown to be an effective tool to reduce PAC-induced mechanical allodynia (Fidanboylu *et al.*, 2011; Kim *et al.*, 2010). Direct analysis of ROS production revealed that ROS levels increased specifically in non-peptidergic DRG neurons and in spinal cord neurons. ROS levels were unchanged in the spinal microglia and astrocytes (Duggett *et al.*, 2016). ROS production can also damage deoxyribonucleic acid (DNA), which may lead to the degeneration/death of sensory neurons (Boyette-Davis *et al.*, 2018).

6.2.2. Paclitaxel and Changes in Ion Channels and Receptors Function

There are several changes in expression/function of ion channels, implicated in PIPN development. For example, sodium channel $Na_v1.7$ was found to be up-regulated following PAC administration in rat and human DRG, especially in the population of peptidergic, CGRP expressing neurons, and in central processes of these cells in the DH. Behavioral signs of PIPN may be attenuated by intrathecal administration of $Na_v1.7$ antagonist *protoxin II* (ProTxII; Li *et al.*, 2018; Zhang & Dougherty, 2014). Recently, it has been shown that inhibition of another sodium channel, $Na_v1.8$, with antagonist *puerarin* could be effective in attenuation of mechanical allodynia and thermal hyperalgesia induced by PAC, and in the reduction of PAC-induced hypersensitivity of DRG neurons (Zhang *et al.*, 2018).

An important role in the development of neuropathic pain plays also potassium channels. Enhanced excitability of primary sensory neurons after PAC treatment may also be a consequence of down-regulation of potassium channels Kir1.1 and Kir3.3 in the DRG. As a result of the down-regulation of potassium channels, rheobase decreased in the population of small-sized DRG neurons (Zhang & Dougherty, 2014).

PAC treatment may induce changes also in the expression of voltage-gated calcium channels; e.g., increased expression of T-type low-voltage-activated calcium channels $Ca_v3.2$ was found in the DRG in the rodent model of PIPN. This increase is associated with the development of spontaneous activity and hyperexcitability of DRG neurons. The development of behavioral hypersensitivity to mechanical stimuli in rats may be prevented by administration of calcium channel inhibitor ML218 hydrochloride (Li *et al.*, 2017).

An important role in the development of PAC-induced hypersensitivity also plays transient receptor potential (TRP) channels—especially TRPV1, which was well known as a key player in the pain sensation and development of neuropathic pain (Nagy *et al.*, 2014; Špicarová *et al.*, 2014b). Hara *et al.* have shown that PAC treatment increased the expression of both TRPV1 mRNA and protein in the DRG neurons, especially in small- and medium-sized neurons (Hara *et al.*, 2013). TRPV1 was also shown responsible for the development of heat hyperalgesia and mechanical allodynia/hyperalgesia in the model of PIPN (Hara *et al.*, 2013; Li *et al.*, 2015a). Also others TRP channels have been reported as contributors to the development of PAC-induced hypersensitivity; e.g., an antagonist of TRPM8 was shown effective in the attenuation of cold hyperalgesia and tactile allodynia; an antagonist of TRPA1 receptors also attenuated the development of mechanical allodynia in the PIPN mice model (Salat & Filipek, 2015). Increased expression of TRPA1 and TRPV4 in the DRG was also reported (Wu *et al.*, 2015).

6.2.3. Paclitaxel and the Role of Glial and Immune Cells

An important role in the development of PIPN plays also activation of glial cells, especially of astrocytes. PAC treatment induces rapid and persistent activation of spinal astrocytes assessed using GFAP labeling (Zhang et al., 2012). In the context of astrocyte activation following PAC treatment, it has been reported down-regulation in the expression of glutamate transporters GLAST and GLT-1 in spinal astrocytes, which are responsible for glutamate clearance in the synaptic cleft (Cata *et al.*, 2006; Zhang *et al.*, 2012). This impairment of the glial glutamate transporters function is mediated by endogenous IL-1β induced trafficking via PKC-signaling pathway (Yan *et al.*, 2014). Analysis performed later showed that PAC had decreased protein expression of both GLAST and GLT-1 in the cell membrane, but increased their expression in the cytosol (Yan *et al.*, 2015b).

Activation of microglia and their contribution to the PIPN is slightly controversial. There are reports showing microglial activation following PAC treatment (Peters *et al.*, 2007; Pevida *et al.*, 2013), in contrast with several reports that have not found any markers (e.g., Iba-1, OX-42 or phosphorylated p38 MAPK) of microglia activation (Zhang *et al.*, 2012; Zheng *et al.*, 2011). One of the explanations of these different findings may be the different experimental protocol used. Whereas both Zheng *et al.* (2011) and Zhang *et al.* (2012) used 8 mg/kg cumulative dose of PAC in four injections and used adult Sprague-Dawley rats, Peters *et al.* (2007) used a high dose of 18 mg/kg of PAC in two injections (36 mg/kg cumulatively), also in Sprague-Dawley rats. Pevida *et al.* (2013) also used a relatively high dose of 10 mg/kg in an only single injection in CD-1 Swiss mice. This suggests that activation of microglia might occur in both rat and murine models more likely after administration of high dose of PAC.

Among glial/immune cells activation, high dose of PAC increased also the expression of nuclear activating transcription factor ATF3, which is a marker of cellular injury, in the DRG neurons, satellite cells, and as well in Schwann cells within the sciatic nerve (Peters *et al.*, 2007). In damaged neurons with increased ATF3 expression has also been observed

alterations in distribution in cytoskeletal proteins, e.g., accumulation of neurofilament NF200[4] protein in medium- and large-diameter sensory neuron cell bodies in DRG (Peters *et al.*, 2007).

An important role in the development of PIPN plays a variety of proinflammatory mediators, released by various cells including glial cells, immune cells, and neurons. These molecules may directly activate/modulate primary afferent fibers, DRG neurons and spinal cord DH neurons (Boyette-Davis *et al.*, 2018). PAC in higher doses may activate satellite glial cells in DRG and may increase the number of CD68 positive, i.e., activated macrophages within the DRG and peripheral nerve (Peters *et al.*, 2007). Activation of satellite glial cells has been shown to increase the expression of TNFβ in the DRG (Wu *et al.*, 2015). Activated macrophages (or mononuclear cells in general) have been shown to enhance release of cytokines, such as TNFα, IL-1β, and CCL2 (Allen *et al.*, 1993; Byrd-Leifer *et al.*, 2001; Zhang *et al.*, 2016). However, PAC alone did not stimulate directly production of TNFα and IL-1β via mRNA transcription, but more likely enhance the production of these cytokines in conjunction with another stimulus (Allen *et al.*, 1993). Activated macrophages infiltrating the DRG may significantly modulate the excitability of sensory DRG neurons via releasing of TNFα and CCL2. Activation of macrophages and cytokine production may be prevented by administration of the macrophage toxin clodronate in liposomes (Zhang *et al.*, 2016).

PAC also induces increased expression of CCL2 in spinal astrocytes and in the DRG neurons. Moreover, DRG neurons following PAC treatment increased the expression of CCR2 receptor for CCL2 (Zhang *et al.*, 2013). The increased level of CCL2 in the spinal cord can stimulate microglial cell activity via CCR2 receptor and thereby potentiate PAC-induced changes. Inhibition of microglia by minocycline, by *i.t.* injection of CCR2 antagonist or by *i.t* injection of CCL2 antibody has been shown to prevented PAC-induced microglial activation and behavioral hypersensitivity (Pevida *et al.*, 2013).

It was shown repeatedly that all of these mediators may directly activate DRG neurons and sensitize, e.g., TRPV1 receptors that play important role in the modulation of nociceptive synaptic transmission under different pathological conditions (Kao *et al.*, 2012; Malek *et al.*, 2015; Nagy *et al.*, 2014; Špicarová *et al.*, 2014a; Špicarová & Paleček, 2010).

6.2.4. PIPN and Genetic Influences

Among other targets of PAC toxicity, mentioned above, there is also another important risk factor. The prevalence of PIPN (and CIPN in general) may be strongly influenced by genetic predispositions. A recent systematic review and meta-analysis identify consistent single nucleotide polymorphism (SNP) changes that could increase the risk of CIPN. For example, PIPN was associated with SNPs in the cytochrome P450 allele *CYP2C8*3* (Cliff *et al.*, 2017).

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[4] NF200 is the high molecular weight (200 kD) neurofilament protein, which forms the structure of the cytoskeleton, and is abundant in axons, especially in medium and large diameter sensory neurons.

7. Toll-Like Receptors and Pain

Toll-like receptors (TLRs) are pattern-recognition receptors well known to be expressed by cells of the innate immune system that recognize microbial pathogens, leading to inflammation and phagocytosis (Milligan & Watkins, 2009). TLRs plays a key role in the non-specific innate immune response via recognition of specific molecular motifs, known as *pathogen-associated molecular patterns* (PAMPs). Each type or TLR is adapted for recognition of distinct PAMPs derived from microorganisms, such as bacteria, viruses, parasites, and fungi. PAMPs including, e.g., endotoxin LPS, bacterial lipoproteins, flagellin, and some types of nucleic acid. As well, TLRs may recognize *dangerous-associated molecular patterns* (DAMPs) released after cells stress and injury. DAMPs including (*I.*) heat-shock proteins (Hsp22, 60, 72, and 90), (*II.*) extracellular matrix degradation products (biglycan, hyaluronan, fibronectin and surfactant protein A), (*III.*) non-histone chromatin-associated *high mobility group box 1 protein* (HMGB1), (*IV.*) oxidized low-density lipoprotein (OxLDL) and (*V.*) small anti-microbial peptides β-defensins (Liu *et al.*, 2012; Miyake, 2007).

TLRs share significant homology with the interleukin-1 receptor family. TLRs contain a large, leucine-rich repeat domain extracellularly and a Toll/Interleukin-1 Receptor (TIR) homology domain in the cytoplasm (Miyake, 2007). TLRs appear to require dimerization either as homo- or heteromers for functional activity. Heteromerization appears to influence substantially the potency of ligand binding (e.g., TLR1/2 and TLR2/6). To date, thirteen TLRs (TLR1–TLR13) have been discovered and described in mammals, but only TLR1–TLR10 has been found in humans (Alexander *et al.*, 2017; Vijay, 2018). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are cell-surface proteins, while other members are associated with membranes of intracellular organelles (Alexander et al., 2017). One exception is TLR7 that is in immune cells localized intracellularly, but it is expressed on the cell surface of DRG neurons, including cell bodies as well as axons. Most of TLRs (with the exception of TLR3) signal through the adaptor protein *myeloid differentiation primary response protein 88* (MyD88). TLR2 and TLR4 also signal via the TIR domain-containing adaptor protein (TIRAP; Alexander *et al.*, 2017; Liu *et al.*, 2012).

TLRs are not only expressed by immune cells; moreover, both neurons and non-neuronal cell types, e.g., astrocytes, microglia, Schwann cells, and oligodendrocytes, in the PNS and CNS, also express TLRs. TLRs activation may contribute to both infectious and non-infectious disorders in the CNS (Liu *et al.*, 2012). In the primary somatosensory DRG neurons has been found expression of TLR4, TLR5, and TLR7 (Ji *et al.*, 2016). Following tissue and nerve injury, TLRs activation induce the activation of microglia and astrocytes in the spinal cord and initiate

immune-like processes, such as the release of inflammatory mediators and phagocytosis. TLRs modulate glial-neuronal communication and create an excitatory positive feedback loop in the pain pathway (Liu *et al.*, 2012; Milligan & Watkins, 2009).

The crucial role of several TLRs has been reported in the modulation of nociceptive synaptic transmission and in the development of chronic neuropathic pain (Christianson *et al.*, 2011; Das *et al.*, 2016; Li *et al.*, 2015a; Li *et al.*, 2014; Liu *et al.*, 2012; Nicotra *et al.*, 2012; Park *et al.*, 2014; Qi *et al.*, 2011; Saito *et al.*, 2010; Sorge *et al.*, 2011; Yan *et al.*, 2015b).

7.1. TLR4 and Paclitaxel-Induced Peripheral Neuropathy

Increasing number of studies has demonstrated that TLR4 plays important role in the modulation of pain and itch (Christianson *et al.*, 2011; Diogenes *et al.*, 2011; Liu *et al.*, 2012; Min *et al.*, 2014) and in the development of PIPN and PAC-induced neuropathic pain (Li *et al.*, 2015a; Li *et al.*, 2015b; Li *et al.*, 2014; Wu *et al.*, 2015; Yan *et al.*, 2015b).

It has been demonstrated that PAC can mimic the activity of prototypical TLR4 agonist—bacterial LPS. Similarly to LPS, PAC activates mouse macrophages in a cell cycle-independent, LPS antagonist-inhibitable manner, suggesting that both LPS and PAC share TLR4/MD-2 complex dependent signaling pathway (Byrd-Leifer *et al.*, 2001; Guha & Mackman, 2001; Kawasaki *et al.*, 2000). However, in contrast to observation in rodents, the effect of PAC on human TLR4 is slightly controversial. There are some evidence that the TLR4 accessory protein MD-2 is a necessary component for PAC-mediated TLR4 activation in mice, but not in human cells (Kawasaki *et al.*, 2001; Resman *et al.*, 2008; Zimmer *et al.*, 2008). Nevertheless, PAC has been shown to be able to activate TLR4 signaling and sensitized TRPV1-mediated capsaicin responses in human DGR and in HEK293 cells co-expressing human TRPV1 and human TLR4 (Li *et al.*, 2015a).

TLR4 activation resulting in the activation of several signaling pathways via two adapter proteins: (*I.*) MyD88 and (*II.*)) TRIF (TIR domain-containing adaptor-inducing interferon-β). Both of them induce the activation of nuclear factor-kappa B (NF-κB) pathway (Takeda & Akira, 2004). In addition to MyD88 and TRIF, there are also three MAPKs signaling pathways involved in TLR4 signaling: (*a*) ERK1/2, (*b*) c-Jun N-terminal kinase (JNK), and (*c*) p38 (Guha & Mackman, 2001).

PI3K/Akt (protein kinase B) pathway may also be involved in signaling downstream to TLR4 and may regulate activation NF-κB (Guha & Mackman, 2001; Ojaniemi *et al.*, 2003).

Activation of mentioned pathways in primary sensory DRG neurons and non-neuronal cells may lead to the regulation of expression and release of proinflammatory cytokines, such as CCL2 and their receptor CCR2 (Zhang *et al.*, 2013), TNFα (Byrd-Leifer *et al.*, 2001; Ledeboer *et al.*, 2007) and IL-1β (Ledeboer *et al.*, 2007). These proinflammatory mediators may subsequently modulate synaptic transmission.

However, it has been also shown that PAC may activate DRG neurons via TLR4 activation and thus modulate synaptic transmission in the spinal cord directly (Li *et al.*, 2015a). This effect of PAC-induced TLR4 signaling is, at least partly, dependent on TRPV1. It was shown that

PAC/TLR4-signaling sensitizes DRG neurons to TRPV1-mediated responses to capsaicin and may lead to sensitization of TRPV1-mediated responses to capsaicin recorded *in vitro* in the spinal cord DH. Cotreatment with TLR4 antagonist LPS-RS prevented this effect of PAC. It was also shown that the number of TRPV1 positive DRG was increased following PAC treatment via a TLR4-dependent mechanism. Cotreatment of rats with antagonist LPS-RS prevented this effect of PAC on the increased expression of TRPV1 in DRG and on the development of mechanical hyperalgesia (Li *et al.*, 2015a), which is a typical symptom of neuropathic pain.

More recently, we reported that PI3K and other serine/threonine kinases (inhibitable with staurosporine, e.g., PKA, PKC or CaMKII), are involved in the TLR4-mediated modulation of TRPV1 sensitivity to agonist capsaicin and that pretreatment with PI3K antagonist wortmannin effectively prevented the PAC-induced mechanical allodynia (Adámek *et al.*, 2019).

TLR4 may be during PIPN activated not only by PAC but also by endogenous substances. Recently, it has been reported that PAC-induce release of an endogenous TLR4 agonist HMGB1 from macrophages. This release is mediated via activation of ROS/p38 MAPK/NF-κB/HAT (histone acetyltransferases) pathway in macrophages (Sekiguchi *et al.*, 2018).

It has been repeatedly showed that spinal TLR4 plays an important role in the inflammation and neuropathic hypersensitivity. However, it seems that its role is *sex-specific* because the involvement of TLR4 has been shown only in male, but not female mice (Sorge *et al.*, 2011). Recently, it has been demonstrated that female sex hormones modulate pain responses also in the animal model of PIPN. Wang *et al.* (2018) reported that the increased expression of proinflammatory cytokines (IL-1β, IL-6 and TNFα; and their respective receptors) induced by PAC was reduced in the DRG of ovariectomized rats. Thresholds of pain responses to mechanical and thermal stimuli appeared to be greater in ovariectomized rats with lack of female sex hormones. Overall, their results indicate that circulating 17β-estradiol and progesterone contribute in females to the modulation and development of neuropathic pain after administration of PAC (Wang *et al.*, 2018), independently of the TLR4 receptors.

8. Pain Associated with Burn Injury

The skin is the body's largest organ system densely innervated by sensory neurons. It functions to maintain homeostasis, including fluid balance, thermoregulation and it also provides an immune barrier. The burn injury of the skin has been described as one of the most painful injuries that can occur. The burn injury may have different causes. Most often cause is THERMAL injury, induced by excessive heat or cold applied to the skin. Other causes include CHEMICAL burns from acid, or alkali exposure, RADIATION burns (sunburn/UV radiation, radiation therapy), and ELECTRICAL burns (Young *et al.*, 2019).

A major burn is a severe injury with a huge impact. It affects survivors and their families physically, psychologically, and emotionally. The burn injury could occur with other trauma, such as brain injury, massive soft tissue loss, amputations, multiple orthopedic injuries, or spinal cord injury, which magnified the final impact (Young *et al.*, 2019).

Burn severity is classified by the extent of affected body surface area and depth of skin injury. According to the depth of injured skin, three types could be distinguished.

(*I.*) SUPERFICIAL PARTIAL THICKNESS BURNS that has been described by older terminology as *first-degree burns* involve partial damage of epidermis. This tissue injury causes the release of proinflammatory mediators in the immediate postburn injury period. These mediators sensitize the nociceptive endings at the area of injury and produce acute inflammatory nociceptive pain. This pain injury is relatively mild to moderate and healing occurs usually within a week (Griggs *et al.*, 2017; James & Jowza, 2017; Young *et al*, 2019).

(*II.*) DEEP PARTIAL THICKNESS BURNS, or by older terminology *second-degree burns*, involve damage of epidermis and a large part of the upper dermis (James & Jowza, 2017; Young *et al.*, 2019).

(*III.*) FULL THICKNESS BURNS represents the severest type of burn injury and involves *third-* and *fourth-degree burns*. This injury involves damage of all layers of epidermis and dermis and extends into the subcutaneous tissue, muscles, nerves or bones. In *deep partial* and *full thickness burns* damage or destruction of peripheral nerves/nerve endings may cause *neuropathic pain* (James & Jowza, 2017; Young *et al.*, 2019).

The most *immediate* and *acute* form of burn injury-induced pain is the already mentioned INFLAMMATORY NOCICEPTIVE PAIN. This acute nociceptive pain is often followed by—in general, two types of pain, which is often experienced latter after burn injury. The first category involves EVOKED and PROCEDURAL PAIN, which occurs with predictable events, such as after a movement or with physical activities and procedures such as dressing changes. This pain is usually short-lived but very high in intensity. The second type is so-called BACKGROUND PAIN,

which is experienced without provocation and it is present even in rest. Usually, it is less intense than evoked/procedural pain. However, it is often constantly present or it can have spontaneous exacerbation without any known reason (Griggs *et al.*, 2017; James & Jowza, 2017).

The treatment of the burns and pain management in burn-injured survivors represent a significant clinical problem. Opioids represent the cornerstone of acute pain treatment, but should not be used as monotherapy. A problem of prolonged-opioid use is *opioid-induced hyperalgesia*, which arises as a consequence of changes in CNS, leading to a decrease in analgesics responsiveness to opioid treatment. Related problem represents also patients with prior history of opioid use. Early identification of opioid-tolerant patients could improve analgesics outcomes. Opioids could be reduced up to 20 % to 30 % by coadministration with *nonsteroidal anti-inflammatory drugs* (NSAIDs[1]; James & Jowza, 2017).

Recent findings also suggest that inhibition of the *voltage-gated sodium channel $Na_v1.7$* in primary sensory neurons could provide promising approach to control pain in patients with burn injury (Cai *et al.*, 2016; Salas *et al.*, 2015; Shields *et al.*, 2012). Therefore, we focused this issue in our experiments, in which we study the effectiveness of $Na_v1.7$ inhibitor protoxin II on spinal cord nociceptive processing following burn injury in an animal model of thermal burn injury (Torres-Perez *et al.*, 2018).

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[1] The mechanism of action of NSAIDs (such as most known aspirin, ibuprofen, acetic and enolic derivates, and many others) is generally thought to be through inhibition of enzymes cyclooxygenase-1 (COX-1) and (COX-2), the enzymes responsible for synthesis of prostaglandins, which contribute to inflammation, pain, and fever. There are two types of NSAIDs: non-selective NSAIDs, which inhibit both COX-1 and COX-2 enzymes, and selective NSAIDs, which inhibit only the COX-2 enzyme. Both selective and non-selective NSAIDs are available for pain treatment. The choice of NSAID is mostly based on the different possible known adverse effects and also cost (Enthoven *et al.*, 2016).

9. The Role of Na$_v$1.7 Sodium Channel in Pain and Nociception

Na$_v$1.7 is a member of *Voltage-Gated Sodium Channels* (VGSCs) family. VGSCs are sodium-selective ion channels present in the plasma membrane of most excitable cells. Sodium channels are comprised of one large pore-forming α subunit, which may be associated with either one or two auxiliary β subunits. α-subunits consist of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6) and a pore-forming loop. Nine members of the VGSCs family have been characterized in mammals, including Na$_v$1.1–Na$_v$1.9 (Catterall *et al.*, 2005; Xu & Yaksh, 2011).

VGSCs are essential for (*I.*) the initial transduction of sensory stimuli by integrating and amplifying of generator potential, (*II.*) control of the electrogenesis and propagation of *"all-or-none"* action potential, and finally (*III.*) neurotransmitter release from sensory neuron terminals at the first synapse within the spinal cord. Therefore, VGSCs are key determinants of the neural excitability. Na$_v$1.2, Na$_v$1.3, and Na$_v$1.5 are expressed exclusively during the embryonic period and subsequently downregulated. However, Na$_v$1.3 can be re-expressed and could contribute to the amplification of subthreshold stimuli following peripheral nerve injury. Na$_v$1.1, Na$_v$1.6, Na$_v$1.7, Na$_v$1.8, and Na$_v$1.9 are all expressed by adult sensory neurons (Bennett *et al.*, 2019). According to the sensitivity to the well-known VGSCs blocker *tetrodotoxin* (TTX) from pufferfish *"fugu"*, VGSCs may be pharmacologically discriminated into *TTX-sensitive* (Na$_v$1.1, Na$_v$1.2, Na$_v$1.3, Na$_v$1.4, Na$_v$1.6, Na$_v$1.7) and *TTX-insensitive* channels (Na$_v$1.5, Na$_v$1.8, Na$_v$1.9; Mattei, 2018).

Several VGSCs are implicated in persistent pain states, including Na$_v$1.3, Na$_v$1.7, Na$_v$1.8, and Na$_v$1.9. Posttranslational modifications, as well as changes in their expression, contribute to the sensitization of sensory neurons in different chronic pain states (Bennett *et al.*, 2019; McKerrall & Sutherlin, 2018; Xu & Yaksh, 2011). Recently, Na$_v$1.1 and Na$_v$1.6 are also suggested as important players in mechanisms of pain (Bennett *et al.*, 2019; Mattei, 2018).

Na$_v$1.7, formerly known also as *peripheral nerve type 1* (PN1) *sodium channel* is one of TTX-sensitive VGSCs. Na$_v$1.7 contributes to the rising phase of the action potential and amplifies subthreshold stimuli. Na$_v$1.7 has a low activation threshold and together with Na$_v$1.6 and Na$_v$1.3 have fast kinetics (Bennett *et al.*, 2019).

Na$_v$1.7 is predominant VGSC expressed in peripheral neurons and nerves (Toledo-Aral *et al.*, 1997). Na$_v$1.7 mRNA has been detected in all types of DRG sensory neurons (Black *et al.*, 1996). Nevertheless, Na$_v$1.7 staining with antibodies showed greater binding in small

nociceptive rather than large size DRG neurons/LTMRs in adult rodents. Moreover, higher Na$_v$1.7 immunoreactivity is positively correlated with the duration of the action potential and negatively correlated with conduction velocity (Djouhri *et al.*, 2003). Approximately 63 % of C-fibers (labeled with marker peripherin) exhibited Na$_v$1.7-immunoreactivity, whereas only 15 % of Aδ-fibers (labeled with marker NF200) was Na$_v$1.7-immunopositive. In the population of nociceptors, Na$_v$1.7 is expressed by ∼65 % of the nonpeptidergic—IB4-positive neurons and by ∼58 % of peptidergic—CGRP-positive neurons. The free nerve endings of unmyelinated fibers within the skin displayed the strong Na$_v$1.7 immunoreactivity (Black *et al.*, 2012). In the CNS is Na$_v$1.7 expressed especially by olfactory sensory neurons (Ahn *et al.*, 2011) and also by *magnocellular neurosecretory neurons* of the *supraoptic nucleus* (Black *et al.*, 2013).

Na$_v$1.7 plays an essential role in normal pain perception in humans. The gene *SCN9A* encodes the α-subunit of Na$_v$1.7. Sequence analysis of the gene *SCN9A* made by Cox *et al.* (2006) has revealed that in humans with *congenital insensitivity to pain* (also known as *congenital analgesia*) there are three distinct homozygous nonsense mutations, which cause *loss-of-function* of Na$_v$1.7 (Cox *et al*l., 2006). Na$_v$1.7 knockout mouse had, similarly to humans with congenital insensitivity to pain, wholly analogous phenotype. In comparison with littermates, knockouts were completely insensitive to painful thermal, chemical and tactile stimuli, while they showed no defects in mechanical sensitivity or movement (Gingras *et al.*, 2014). Moreover, global deletion of Na$_v$1.7 in rodents is also responsible for anosmia—the inability to detect odors (Gingras *et al.*, 2014; Weiss *et al.*, 2011). It is related to high Na$_v$1.7 expression and their key role in excitability of the olfactory sensory neurons (Ahn *et al.*, 2011).

It has been mentioned that Na$_v$1.7 plays an essential role in the perception of acute nociceptive pain. Paradoxically, mutations of the same gene *SCN9A* causes, on the one hand, CONGENITAL INSENSITIVITY TO PAIN, while, on the other hand, other specific *"gain-of-function mutations"* of the same gene cause distinct rare human pain syndromes, such as INHERITED ERYTHROMELALGIA (IEM) (Bennett *et al.*, 2019). IEM, also known as a *man-on-fire syndrome*, is characterized by severe pain and erythema of the extremities. Currently, it is known about 26 mutations linked to IEM (Bennett *et al.*, 2019). For example, the firstly reported mutations linked to IEM are caused by two-point substitution when isoleucine 848 is changed to threonine (I848T) and leucine 858 is changed to histidine (L858H). This substitution produces a hyperpolarizing shift in activation and slower deactivation of Na$_v$1.7. These mutations also cause an increase in amplitudes or the Na$_v$1.7-mediated currents in response to small and slow depolarization (Cummins *et al.*, 2004).

Moreover, Na$_v$1.7 is also involved in other persistent pain syndromes or diseases, e.g., (*a*) PAROXYSMAL EXTREME PAIN DISORDER, known also as familial rectal pain, is another type of inherited disease. Currently, it is known about eight mutations linked this disorder (Bennett *et al.*, 2019); (*b*) SMALL-FIBER NEUROPATHY (SFN) is accompanied by degeneration of the unmyelinated and thinly myelinated axons. SFN may be induced secondary to diabetes (*painful diabetic neuropathy*) or by *chemotherapy* (CIPN). In about 50 % of cases, SFN is described

as idiopathic, without an obvious cause. In these cases, SFN may be a consequence of several *gain-of-function* mutations—currently, it is known about 10 mutations linked to SFN (Bennett *et al.*, 2019; Li *et al.*, 2018). In the rat model of PIPN has been demonstrated that PAC induces $Na_v1.7$ upregulation in CGRP-positive DRG neurons and in the central process of these cells in the superficial dorsal horn. Moreover, $Na_v1.7$ was also colocalized with CGRP and with TRPV1 in human DRGs. PAC-treatment induces an enhancement of Nav1.7-mediated currents in the rat model. A selective $Na_v1.7$ channel blocker *protoxin II* (ProTxII) suppressed spontaneous action potential firing in DRG neurons originating from animals with PIPN. Intrathecal administration of ProTxII also attenuated behavioral signs of PIPN (Li *et al.*, 2018).

Shields *et al.* proposed that the $Na_v1.7$-mediated currents contribute also to the hyperexcitability in sensory neurons damaged by burn injury (Shields *et al.*, 2012).

Recently, the effectiveness of $Na_v1.7$ selective blocker ProTxII has been demonstrated also in attenuation of spinal nociceptive processing following burn injury (Torres-Perez *et al.*, 2018).

$Na_v1.7$ plays a key role in excitability of sensory neurons and therefore it represents a promising target for the development of new pain therapy. Although $Na_v1.7$ has been subjected to an intense investigation, to date, no $Na_v1.7$-selective drugs have been developed for the clinic use (Chew *et al.*, 2019).

10. The Role of Inhibitory Synaptic Processing in Pain And Nociception

It was already mentioned in SECTION 2.3 (p. 15) that inhibitory synaptic control in the DH of the spinal cord plays an absolutely essential role in the maintenance of normal nociceptive transmission and somatosensory perception. Somatosensory processing within the DH requires the precise interaction of GABAergic and glycinergic interneurons with other types of neurons and non-neuronal cells in the DH, as well with central terminals of primary sensory neurons through a *postsynaptic* and *presynaptic* mechanism (Zeilhofer, 2008; Zeilhofer *et al.*, 2012). It has been shown experimentally that pharmaceutical ablation of GABAergic and/or glycinergic component of neurotransmission in the DH may mimic symptoms of neuropathic and inflammatory pain (Baba *et al.*, 2003; Sherman & Loomis, 1995; Sivilotti & Woolf, 1994; Yaksh, 1989). Moreover, it has been demonstrated that a reduction of synaptic inhibition—*disinhibition* occurs naturally in the course neuropathic and inflammatory diseases/pain states (Coull *et al.*, 2005; Harvey *et al.*, 2004; Imlach *et al.*, 2016; Lorenzo *et al.*, 2014; Moore *et al.*, 2002; Muller *et al.*, 2003; Zhou *et al.*, 2012). It seems that disinhibition contributes to the development of neuropathic and inflammatory pain states much more than the excessive activity of primary sensory afferent themselves (Zeilhofer, 2005), therefore focusing on positive modulation and restore of reduced inhibitory component seems to be an interesting target for the development of new treatment strategies (Zeilhofer *et al.*, 2018).

10.1. Molecular Components of Inhibitory Control in the Dorsal Horn

There are two amino acids, GABA (γ-aminobutyric) and glycine, which mediate *fast synaptic inhibition* in the CNS and play the key role in normal sensory processing. The fast synaptic inhibition is mediated via activation of ionotropic $GABA_A$ and glycine receptors (GlyRs) belonging to the *neurotransmitter-gated ion channel of the Cys-loop superfamily*, together with serotonin and nicotinic acetylcholine receptors (Lynch, 2009; Olsen & Sieghart, 2008).

Both $GABA_A$ and GlyRs are composed of five subunits (each with four transmembrane domains), that forms intrinsic anion selective channel permeable for chloride and, to a lesser extent, bicarbonate ions through the plasma membrane. Activation of both receptors usually leads to inhibition of the neurons via hyperpolarization of cell membrane and impairment of

propagation of excitatory synaptic potentials (Lynch, 2009; Olsen & Sieghart, 2008; Zeilhofer *et al.*, 2012).

10.1.1. GABA Receptors

Altogether, nineteen $GABA_A$ receptors subunits have been reported in mammals; $\alpha(1-6)$, $\beta(1-3)$, three $\gamma(1-3)$, δ, $\rho(1-3)$, ϵ, π and θ (Olsen & Sieghart, 2008). This repertoire of subunits forms about 50 different combinations and therefore $GABA_A$ represents the most diverse family of neurotransmitter receptors in mammalian CNS. The majority of $GABA_A$ receptor subunit reveal a widespread distribution within the spinal cord, except $\alpha1$ and $\alpha5$ subunits that are most abundant in intermediate zone (laminae III–VIII) and $\alpha2$ subunit expressed mostly in superficial dorsal horn (laminae I/II) and in somatic and preganglionic motoneurons (Bohlhalter *et al.*, 1996). Heterogeneity of subunit composition in lamina II has also been reported among inhibitory (IN–INs) and excitatory interneurons (EX–INs). While GABAergic IN–INs (but not glycinergic) displayed slower kinetics of evoked or miniature inhibitory postsynaptic currents (eIPSC, resp. mIPSC)[1] determined by the $\alpha3$ subunit, the IPSCs recorded from putative EX–INs displayed fast kinetics determined by $\alpha1$ and $\alpha2$ subunits (Labrakakis *et al.*, 2014).

Several modulatory drugs positively modulate $GABA_A$ receptors including *barbiturates*, *benzodiazepines*, and *alcohol*. Bicuculline and gabazine are the most commonly used antagonists used in research for inhibition of $GABA_A$ receptors (Zeilhofer *et al.*, 2012).

Among fast inhibitory transmission, mediated by $GABA_A$ receptors, they may also mediate sustained tonic inhibition as a response on ambient concentration of GABA. These $GABA_A$-mediated tonic events have been termed *slow synaptic inhibition* (Olsen & Sieghart, 2008).

Except for neurons, the expression of functional $GABA_A$ receptors has been also found in the spinal astrocytes that indirectly participate in synaptic processing. Furthermore, only a subpopulation of normal astrocytes expresses $GABA_A$ receptors whereas all astrocytes that become reactive following nerve injury, application of IL-6 or fibroblast growth factor express $GABA_A$ (Hosli *et al.*, 1997), which may contribute to the development of chronic pain states (Ji *et al.*, 2016). However, the role of non-neuronal $GABA_A$ receptors is not fully understood.

Moreover, among ionotropic $GABA_A$ receptors, there is another group of GABA receptors, involved in the regulation of synaptic transmission—*metabotropic $GABA_B$ receptors*. $GABA_B$ are dimers formed from the two seven-transmembrane G-protein subunits termed $GABA_{B1}$ and $GABA_{B2}$. $GABA_B$ are located widespread in the CNS and regulate both pre- and post-synaptic activity. On presynaptic endings, $GABA_B$ serves as an autoreceptor and/or heteroreceptor that regulate the transmitter release of GABA and/or all other neurotransmitters by suppressing neuronal Ca^{2+}-conductance through inhibition of high-voltage activated channels of the N-type ($Ca_v2.2$) or P/Q-type ($Ca_v2.1$). On postsynaptic site, $GABA_B$ activation produces

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[1] Evoked currents are induced by electric stimulation (by stimulation electrode), whereas miniature currents recorded in the presence of tetrodotoxin (TTX), to inhibit action potential mediated synaptic release, represents spontaneous action potential–independent release.

an increase in membrane potassium (K^+) conductance and associated slow neuronal hyperpolarization/late inhibitory postsynaptic current via activation of inwardly rectifying K^+ channels, GIRK or Kir3 (Bettler *et al.*, 2004; Bowery *et al.*, 2002).

10.1.2.   Glycine Receptors

Strychnine-sensitive GlyRs represent second and an equally important component of *fast inhibitory transmission* in the mammalian nervous system. Antagonist strychnine is a useful tool for differentiation of GlyRs from both $GABA_A$ receptors and also from glutamate NMDA receptors, where glycine serves as a co-agonist of L-glutamate, required for full activation of NMDA channel (Erreger *et al.*, 2004; Zeilhofer, 2005).

GlyRs may assembly as homopentamers of α-subunits in immature CNS, while in adult CNS, GlyRs are typically heteromers of 2α and 3β subunits. It has been identified four α (α1–4) and one β (β1). Further variability originates from post-transcriptional modification/alternative splicing of α2, α3, and α4 subunits. GlyRs are located both post- and presynaptically, where they modulate neurotransmitter release (Lynch, 2009). A scaffold protein *gephyrin* is essential for the proper clustering of GlyRs at postsynaptic densities via direct interactions between β subunit and microtubules (Baer *et al.*, 2003).

In comparison with $GABA_A$ receptors widely expressed throughout the mammalian CNS, GlyR reveals more specific distribution. A high density of GlyRs is both in the dorsal and the ventral horn of the spinal cord (Zeilhofer *et al.*, 2012). GlyR α3 subunits are expressed predominantly in the substantia gelatinosa (lamina II), where most nociceptive afferents terminate and make synaptic connections with projection and/or interneurons. GlyRs α3 subunit has been shown responsible for PGE_2-induced inhibition of glycinergic transmission, which suggests their important role in the pathology of pain (Harvey *et al.*, 2004).

10.1.3.   GABA and Glycine: Synthesis, Storage and Reuptake

Inhibitory amino acids are synthesized by inhibitory interneurons (IN–INs) that represents about one-third of all neurons in laminae I–II and $\sim$40 % of those in lamina III (Todd, 2015). Glycine immunoreactivity within the superficial DH (laminae I–III) is significantly restricted to GABAergic cells, which suggest that majority of glycine-positive IN–INs also co-release GABA, whereas the others are purely GABAergic (Todd, 2010; Todd *et al.*, 1996).

GABAergic neurons synthesized GABA from L-glutamate (glutamic acid) by the enzyme glutamic acid decarboxylase (GAD) that exists in two isoforms GAD65 and GAD67. GABA is loaded into presynaptic vesicles by the vesicular GABA transporter VGAT[2]. VGAT is also responsible for transport of glycine into the vesicles. VGAT is frequently used as a marker of IN–INs, while GAD65 or GAD67 are specific only for the population of GABAergic IN–INs (Zeilhofer *et al.*, 2012).

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[2] VGAT (gene *Slc32a1*) is also known as vesicular inhibitory amino acid transporter VIAAT.

There are four major GABA transporters—GAT1, GAT2, GAT3, and BGT1[3], which removes GABA after synaptic release from synaptic cleft and are responsible for termination of GABAergic inhibitory postsynaptic currents and recycling of GABA. GAT1 and GAT3 are expressed by both neurons and astrocytes (Zeilhofer *et al.*, 2012; Zhou & Danbolt, 2013).

For the accumulation of glycine intracellularly, glycinergic IN–INs express plasma membrane glycine transporter GlyT2. Co-expression of GlyT2 with VGAT is used as a marker of the glycinergic population of IN-INs. GlyT2 is located in terminals of the glycinergic axons, where is responsible for uptake of released glycine from the synaptic cleft. Moreover, GlyT1 also exists however it is not restricted only to glycinergic population or area of glycinergic innervation (Zeilhofer *et al.*, 2012).

10.2. Mechanisms of Disinhibition

It has been demonstrated repeatedly by several groups that synaptic inhibition in the DH becomes diminished in animal models of neuropathic (Moore *et al.*, 2002) and inflammatory pain (Muller *et al.*, 2003).

Several mechanisms have been proposed responsible for this disinhibition, including, e.g.: (*I.*) death of IN–INs (Moore *et al.*, 2002); (*II.*) reduced afferent excitatory drive to IN–INs (Polgar & Todd, 2008); (*III.*) changes in normal GABA and glycine synthesis/function (Lorenzo *et al.*, 2014); and (*IV.*) changes in membrane properties of DH neurons (Coull *et al.*, 2003).

It also seems that disinhibition may uncover a novel polysynaptic low-threshold input onto lamina I neurons, suggesting that inhibitory pathway normally suppresses a preexisting pathway responsible for abnormal pain sensation such as allodynia (Takazawa & MacDermott, 2010). An important role in the disinhibition could play also endocannabinoid system. Endocannabinoids, such as AEA or 2-AG, and drugs acting on cannabinoid receptor CB_1 are generally believed to suppress nociception and pain perception. However, disinhibition could occur also after intense nociceptive stimulation (induced, e.g., by subcutaneous capsaicin injection) in the absence of inflammation or neuropathy. It was shown that endocannabinoids via activation of CB_1 receptor located on DH IN–INs diminishes the inhibitory control and thereby endocannabinoids facilitate nociception (Pernia-Andrade *et al.*, 2009).

Despite numerous studies published on the topic of disinhibition, it is still not clear which of these changes and mechanisms contributes to the development and maintenance of chronic pathological pain states after nerve injury, inflammation or chemotherapy.

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[3] BGT1 also known as betaine-GABA transporter 1 is a member of the Betaine/Carnitine/Choline Transporter (BCCT) family.

10.2.1. Disinhibition and Nerve Injury-Induced Neuropathy

Several authors reported that neuronal apoptosis is responsible for the loss of normal inhibition in the DH after peripheral nerve or spinal cord injury (Ibuki *et al.*, 1997; Meisner *et al.*, 2010; Moore *et al.*, 2002; Scholz *et al.*, 2005). The authors conclude so because of decreased GABA and GABA synthesizing enzyme GAD65/67 immunoreactivity, increased apoptotic markers, and reduced inhibitory postsynaptic currents. For example, it has been reported that nerve injury leads in CCI and SNI models to significant decrease in the amplitude and duration of eIPSC, which is evoked by electrical stimulation of dorsal roots, in the DH neurons (Moore *et al.*, 2002; Scholz *et al.*, 2005). The frequency of miniature GABA$_A$ IPSCs (mIPSCs), but not the amplitude of these currents was changed after both CCI and SNI. Surprisingly no changes both in evoked and miniature IPSC was observed after SNT (see FIGURE 3.1: Animal models of the peripheral nerve injury; p. 28). Moreover, SNI decreased DH levels of GAD65 (Moore *et al.*, 2002). These findings suggest presynaptic mechanisms involving the IN–INs. This is consistent with immunohistochemical evidence that no reduction of GABA receptors in the DH occur following nerve injury (Polgar & Todd, 2008).

However, other studies refute these findings for neuronal apoptosis and have not shown any significant changes in the number of GABA-immunoreactive neurons in laminae I–III following CCI or SNI (Polgar *et al.*, 2003; Polgar & Todd, 2008) and apoptotic cells in the DH were microglia, rather than neurons (Polgar *et al.*, 2005). Nevertheless, all of these studies reported clear behavioral signs of neuropathic pain, even without loss/apoptosis of GABAergic neurons (Polgar *et al.*, 2005; Polgar *et al.*, 2003; Polgar & Todd, 2008). However, this issue is still controversial (Todd, 2015).

An alternative explanation for disinhibition suggests that the synthesis of GABA in GABA-ergic neurons is affected, which leads to the reduction of transmitter release. Moore *et al.* (2002), who reported a 20–40 % decrease in the GAD65 level after CCI and SNI, but not in the GAD67, support this suggestion. Detailed analysis carried by Lorenzo *et al.* (2014) showed that CCI induced transient loss of GAD65 immunoreactive terminals was greatest in lamina II around 3–4 weeks after injury.

The important role could also play postsynaptic changes. Postsynaptic effect of GABA and glycine is largely dependent on the chloride equilibrium potential. Ionotropic inhibition requires the potassium-chloride exporter KCC2 to maintain low intracellular Cl$^-$ level. Coull *et al.* (2003) found that CCI decrease the expression of KCC2, which leads to the disruption of anion homeostasis in laminal I DH neurons. This shift in chloride equilibrium cause that normally inhibitory anionic currents switch to be excitatory. Local blockade or knock-down of the KKC2 in naive animals produces similar changes such as nerve injury (Coull et al., 2003). Following nerve injury, activated microglia may via the ATP/P2X$_4$-dependent mechanism triggered the release of BNDF. BDNF from activated microglia induces via neuronal TrkB receptor down-regulation of KCC2 and accumulation Cl$^-$ ions in the DH neurons (Bonin & De Koninck, 2013; Coull *et al.*, 2005). KCC2 down-regulation after CCI or L5/L6 SNL may also be mediated by

the NMDA receptor-dependent mechanism. It has been shown that increased NMDA receptor activity by nerve injury can impair synaptic inhibition through an increased level of Ca^{2+} and by *calpain*[4]-mediated KCC2 proteolysis in the spinal cord DH. The decrease in KCC protein level was prevented by calpain inhibitor *calpeptin* and by NMDA receptor blocker AP5 that also reverse mechanical allodynia (Zhou *et al.*, 2012).

However, the proposed alterations in KCC2 and chloride equilibrium would not explain the reduction of mIPSC frequency that was reported after nerve injury (Moore et al., 2002; Scholz *et al.*, 2005; Todd, 2015). Therefore, it seems likely that both presynaptic and postsynaptic alterations are required for the disinhibition establishment.

One of the other mechanisms involved in the disinhibition following nerve injury is *reduced excitatory drive to the spinal IN–INs.* Leitner *et al.* (2013) showed that CCI reduce the frequency, but not the amplitude of mEPSC recorded from EGFP-labeled GABAergic neurons in spinal cord lamina II. The number of excitatory synapses on GABAergic neurons was analyzed, however, no changes in the density or morphology of dendritic spines were found. Analysis of paired-pulse ratio (PPR) of eEPSC showed decreased release probability at excitatory synapses between Aδ/C-fibers and GABAergic neurons (Leitner *et al.*, 2013). In summary, these data suggest that reduced release probability on IN–INs may contribute to the phenomenon of disinhibition rather than morphological changes or direct loss of excitatory synapses.

In addition, *glycinergic dysfunction* may be significantly involved in the development of neuropathic pain, as well as the disruption of GABAergic inhibition. Imlach *et al.* (2016) demonstrated loss of glycinergic input in a specific population of spinal cord excitatory interneurons, radial cells, in a rat model of PNL. The amplitude of eIPSC was greatly reduced in radial neurons together with increased PPR. They reported also a reduction in the frequency of both spontaneous (sIPSC) and mIPSC, which suggest reduced glycine release probability and thus presynaptic mechanism. Another important finding reported in this study is that loss of glycinergic input following PNL is not due to PGE$_2$-induced activation of EP2 receptor and phosphorylation of GlyR α3 subunit, which plays important role in inflammatory pain (see below SECTION 10.2.2), but the immature GlyR α2 subunit was found increased following nerve injury (Imlach *et al.*, 2016).

10.2.2. Disinhibition and Inflammatory Pain

Loss of glycinergic and GABAergic inhibition may also occur during inflammation (Zeilhofer, 2008).

A crucial role in the modulation of pain during inflammation plays PGE$_2$, which may facilitates the activation of ion channel involved in nociception, e.g., TRPV1 (Moriyama *et al.*, 2005) or TTX-resistant sodium channels (England *et al.*, 1996; Rush & Waxman, 2004). However, locally released PGE$_2$ has been shown to inhibit the function of glycine receptors, leading to loss of local inhibition in the DH neurons. PGE$_2$ via PKA-mediated signaling reversibly reduced

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[4] Calpain is Ca^{2+}-dependent protease

the amplitudes of glycine receptor-mediated eIPSC; as well, spontaneously occurring glycinergic mIPSC were reduced in their amplitudes, but not in frequency. These data indicate mainly postsynaptic site of PGE_2 action (Ahmadi *et al.*, 2002). Later, the role of GlyR isoform that contains $\alpha 3$ subunit was identified in this PGE_2-mediated inhibition of glycinergic transmission. PGE_2 via PKA-dependent phosphorylation induces inhibition of GlyR $\alpha 3$ subunit, which was shown responsible for the development of central thermal and mechanical hypersensitivity under the inflammatory conditions (Harvey *et al.*, 2004). Prostaglandin receptor EP2 was identified as a key signal element in this PGE_2 signaling. Whole-cell patch-clamp recording from spinal cord slices of EP2 deficient mice showed that reduction of glycinergic IPSC is exclusively mediated through EP2 receptor. Moreover, EP2 knockout mice completely lack spinal PGE_2-evoked hyperalgesia (Reinold *et al.*, 2005). The promising strategy to alleviates inflammatory pain may be a selective potentiation of phosphorylated GlyR. It has been demonstrated that 2,6-DTBP[5] reverses PGE_2-mediated disinhibition through a specific interaction with heteromeric $\alpha\beta$GlyRs containing phosphorylated $\alpha 3$ subunit (Acuna *et al.*, 2016).

Peripheral inflammation may induce long-lasting sensitization in the CNS. It was demonstrated in a model of long-lasting inflammation (induced by CFA) that spontaneously occurring glycinergic mIPSCs recorded in lamina I were not affected in amplitude or kinetics, however, the frequency of mIPSCs was significantly reduced when compared with neurons from non-inflamed animals. These data suggest that a reduced glycinergic inhibitory control occurs via a presynaptic mechanism (Muller *et al.*, 2003). The similar decrease in the frequency of sIPSC was reported following CFA treatment (Takazawa *et al.*, 2017). Moreover, Takazawa et al. showed that GABA dominantly inhibits EX–IN and projection neurons of lamina I and II_o (NK_1R-expressing neurons), while those in deeper laminae II_i and III (PKCγ-expressing neurons) are predominantly inhibited by glycine. However, following CFA injection into mouse hind paw was decreased in the frequency of sIPSC accompanied by an increase in GABA dominance in lamina II_i. This shift was not accompanied by a change in the number of synapses or by the change of postsynaptic GlyR $\alpha 1$ subunits expression (Takazawa *et al.*, 2017).

It has also been shown that long-term potentiation (LTP) of glycinergic synapses on inhibitory GABAergic IN–INs may be involved in the phenomenon of disinhibition. LTP of glycinergic synapses occurs after exposure to the inflammatory cytokine IL-1β and it was induced experimentally *in vivo* in a model of formalin-induced peripheral inflammation. This LTP potentiates glycinergic synapses on IN–INs DH neurons and thus produce disinhibition (Chirila *et al.*, 2014).

Recently has been reported that CFA-induced peripheral inflammation may similarly to nerve injury induce BDNF-dependent KCC2 down-regulation in spinal cord neurons. However, the mechanism reported by Lalisse *et al.* (2018) is different when compared with the mechanism reported after nerve injury in which activated microglia produce BDNF (Coull *et al.*, 2005). Lalisse *et al.* reported that ATP-gated purinergic receptor $P2X_4$ is upregulated in DGR during

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[5] 2,6-Di-*tert*-butylphenol, a nonanesthetic propofol derivate.

long-lasting peripheral inflammation. They also showed that $P2X_4$ control calcium influx into the $P2X_4$-expressing DRG neurons and release of BDNF in the DH. Then, the released BDNF may via TrkB on IN–INs down-regulate KCC2 and thus disrupt chloride equilibrium (Lalisse *et al.*, 2018). The role of BDNF/TrkB signaling and decreased KCC2 expression has also been reported in the model of acute inflammatory pain, 5 minutes after the plantar injection of formalin in rats (Tsuruga *et al.*, 2016).

10.2.3. Disinhibition and Paclitaxel-Induced Peripheral Neuropathy

The mechanism of PIPN and CIPN, in general, are extensively studied, however, little is known about how PAC/chemotherapy affects inhibitory synaptic control within CNS. To our knowledge, the first evidence that PAC impairs inhibitory synaptic transmission in the spinal cord DH was reported by Chen *et al.* (2014). They showed that PAC-treatment up-regulates protein levels, but not mRNA levels of Na^+-K^+-$2Cl^-$ *cotransporter-1* (NKCC1) in the spinal cord. NKCC1 up-regulation caused a depolarizing shift in GABA reversal potential of DH neurons and thus significantly reduced GABA-mediated hyperpolarization in lamina II neurons. Inhibition of NKCC1 with *bumetanide* reversed the PAC effect on GABA-mediated hyperpolarization. Intrathecal bumetanide, as well, significantly attenuated PAC-induced tactile allodynia and hyperalgesia. The authors suggest that this effect of PAC is mediated by the disruption of intracellular NKCC1 trafficking by interfering with microtubule and associated motor proteins; PAC-induced stabilization of β-tubulin and an increase in total plasma membrane NKCC1 protein level, while reducing the cytosolic fraction of NKCC1 (Chen *et al.*, 2014b).

PAC-induced disinhibition may also be caused by affecting of GABA transporter GAT-1 and tonic GABAergic inhibition. Yadav *et al.* (2015) showed that in rats with PIPN, the protein expression of GAT-1, expressed in presynaptic terminals and astrocytes, was increased while GAT-3, expressed selectively by astrocytes, was decreased. This, in turn, increases the efficiency of GABA uptake and attenuated tonic GABAergic inhibition, which was ameliorated by GAT-1, but not by GAT-3 blocker. Intrathecal application of GAT-1 inhibitor NO-711 significantly attenuated mechanical allodynia and thermal hyperalgesia in a behavioral experiment (Yadav *et al.*, 2015). Systemic *i.p.* GAT-1 blocker NO-711 administration was also reported as effective in the murine model of PIPN, in which prevented PAC-induced thermal hyperalgesia and cold allodynia, and alleviates established PAC-induced thermal hyperalgesia and cold allodynia (Masocha & Parvathy, 2016).

Moreover, GAT-1 expression (mRNA) was found increased following PAC-treatment also in the *anterior cingulate cortex*—the area involved in the pain perception. This increase may produce disinhibition at the synapses via increased GABA uptake efficiency (Masocha, 2015).

It was already mentioned above, that TLR4 may play an important role in the development of PIPN and that PAC can mimic LPS activity. Yan *et al.* (2015a) reported that activation of TLR4 by LPS reduces GABAergic synaptic activity through both postsynaptic and presynaptic mechanism: (*I.*) LPS cause the release of IL-1β from activated microglia, which

in turn suppresses via PKC-signaling postsynaptic GABA receptors function and reduces both frequency and amplitude of GABAergic mIPSCs (Yan *et al.*, 2015a); (*II.*) GABA synthesis may be reduced following LPS stimulation via IL-1β, which was previously shown to suppress glutamate transporters GLT-1 and GLAST (Yan *et al.*, 2015a; Yan *et al.*, 2014). Their suppression, in turn, leads to the deficiency of glutamine supply resulting in an attenuation of the *glutamate-glutamine cycle*-dependent GABA synthesis (Yan *et al.*, 2015a).

Part II.

EXPERIMENTAL WORK

11. Aims of the Ph.D. project

The aim of my Ph.D. project and experiments in which I participated was to investigate the mechanisms of development of different pain states. The main attention was paid to study the mechanism of *paclitaxel-induced neuropathic pain*, *burn injury-induced pain*, *pain induced by peripheral inflammation* and *neuropathic pain induced by chronic constriction of the sciatic nerve*. Although, these pain states have entirely different causes and mechanisms of development, one aspect of our research was the same.

This aspect is the modulation of the nociceptive information at the spinal cord level. All pain states studied have a cause in damage/injury of peripheral tissues, which leads in all cases to aberrant activity of primary afferent sensory fibers and subsequently aberrant function of nociceptive synaptic transmission at the spinal cord level. In addition, the low concentration of paclitaxel penetrates the spinal cord and therefore may modulate the nociceptive processing directly in the dorsal horn.

The spinal cord dorsal horn represents the first site of synaptic processing in the pain pathway. It represents the main site of nociceptive modulation, where the character and strength of the signal from the periphery to higher centers can be modified—either amplified or even completely attenuated according to current conditions. Therefore, the dorsal horn becomes a promising source or possible targets for improvement of pain management. However, to identify new therapeutic approaches for the treatment of these painful conditions, it is first necessary to understand the changes and processes that occur in the dorsal horn. Therefore, we examined the following experimental questions:

I.) Which is the role of TLR4 and TRPV1 receptors in the paclitaxel-induced neuropathic pain? Which intracellular pathways and kinases are involved in the signaling? How does pharmacological targeting of these pathways help to alleviate pain *in vivo*?

II.) How do different pain states affect/diminish the inhibitory synaptic transmission in the dorsal horn? The aim is to describe and compare the development of disinhibition in the models of paclitaxel-induced peripheral neuropathy, peripheral inflammation and in a chronic constriction injury of the sciatic nerve.

III.) How does the $Na_v1.7$ receptor inhibition by antagonist protoxin II affect the spinal nociceptive signaling in the model of burn injury-induced pain?

IV.) How does the anandamide precursor 20:4-NAPE modulate the nociceptive synaptic transmission under the inflammatory conditions and which role plays CB_1 receptors in this process?

The results of experimental work have been published in five experimental papers (see Appendix, p. 153). The experimental part of this doctoral thesis consists of a presentation and a discussion of the main findings of either published and in part unpublished data.

12. Methods

12.1. Statement of Ethical Consideration

All experiments were approved by the local Institutional Animal Care and Use Committee and were consistent with the guidelines of the International Association for the Study of Pain, EU Directive 2010/63/EU for animal experiments and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were designed to minimize animal discomfort and to reduce the number of animals needed for statistical analysis.

12.2. Animals

Adult male mice C57BL/6 weighting 25 to 30 g, adult male transgenic mice VGAT-ChR2-eYFP line 8[1] and male P19–P21 or adult male Wistar rats were used in our experiments. The animals were housed in separate clear plastic cages with soft bedding, free access to food and water and maintained on 12 hours light/12 hours dark cycle at room temperature-controlled conditions.

12.3. Chemicals

All basic chemicals and drugs used for the preparation of the dissection, recording and intracellular solution were of analytical grade and purchased from Sigma-Aldrich (Prague, Czech Republic). GABA$_A$ receptor antagonist bicuculline methiodide, TRPV1 antagonist AMG9810, and carrageenan for induction of peripheral inflammation were purchased from Sigma-Aldrich. Tetrodotoxin citrate (TTX), staurosporine, protoxin II (ProTxII), SB366791 and PF514273 were purchased from Tocris Bioscience (Bristol, UK). Anandamide and 20:4-NAPE were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Paclitaxel (PAC) used for electrophysiology, capsaicin, wortmannin (WMN), LY-294002 hydrochloride (LY), staurosporine (STAURO), and anandamide (AEA) were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich), which had a concentration <0.1 % in the final solution. 20:4-NAPE was dissolved in chloroform, which had a concentration <0.1 % in the final solution. LPS (Sigma-Aldrich) was dissolved in re-distilled water. Paclitaxel Mylan (Oncotec Pharma Produktion, Germany) was used in the behavioral study.

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[1] Also known as B6.Cg-Tg(Slc32a1-COP4*H134R/EYFP)8Gfng/J; The Jackson Laboratory; Stock No: 014548

12.4. Spinal Cord Slice Preparation for Electrophysiological Experiments

The same experimental protocol was used for both adult mice and juvenile rats spinal cord slices preparation. Laminectomy was performed under deep anesthesia with 3% isoflurane (Forane®, Abbott) and the lumbar spinal cord was removed and immersed in oxygenated, ice-cold (~4 °C), dissection solution containing (in mM) 95 NaCl, 1.8 KCl, 7 MgSO$_4$, 0.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 26 NaHCO$_3$, 25 D-glucose and 50 sucrose. Animals were killed by subsequent medulla interruption and exsanguination. The spinal cord was fixed to a vibratome stage (VT 1200S, Leica, Germany) using cyanoacrylate glue in a groove between two agar blocks. Acute transverse slices 300 µm thick were cut from L4–L5 segments and incubated in the dissection solution for 30 min at 35 °C. Slices were then stored in a recording solution at room temperature (21–24 °C) and allowed to recover for at least 1 h before the electrophysiological experiments. The recording solution contained (in mM) 127 NaCl, 1.8 KCl, 1.2 KH$_2$PO$_4$, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 26 NaHCO$_3$, and 25 D-glucose. For the electrophysiological measurement, slices were transferred into a glass-bottomed recording chamber perfused continuously with the recording solution at room temperature at a rate of ~2ml/min. All extracellular solutions were saturated with carbogen (95% O$_2$, 5% CO$_2$) during the whole experiment.

12.5. Animal Models Used in Our Experiments

12.5.1. Paclitaxel-Induced Peripheral Neuropathy Model

We used two experimental protocols to induce PIPN. In some experiments, adult male mice or rats were treated intraperitoneally (*i.p.*) by a single high dose PAC application (8 mg/kg) of PAC (Paclitaxel Mylan) on Day 0 after the control behavioral measurement. In another set of experiments, we used a dosage of 2 mg/kg (*i.p.*) every other day for a total of four injections (days 0, 2, 4 and 6). Final cumulative dose was 8 mg/kg. Original clinically used stock solution of PAC was diluted with sterile saline (0.9% NaCl) to 2 mg/ml from original concentration 6 mg/ml (in 1:1—macrogolglycerol ricinoleate (Kolliphor EL):ethanol).

In some experimental groups, pretreatment with WMN (0.6 mg/kg, *i.p.*) or STAURO (0.1 mg/kg, *i.p.*) had preceded 1 hour before the PAC treatment. A stock solution of WMN (12.5 mM in DMSO) and STAURO (2.5 mM in DMSO) were diluted in sterile saline solution. The PAC-only treated animals received a sham injection (DMSO as a vehicle) 1 hour before the PAC, corresponding to the other experimental situations.

Mechanical paw withdrawal threshold (see Section 12.6, p. 81) was tested before (on Day 0), during (on Days 2, 4, 6) and after PAC treatment (on Day 7).

12.5.2. Model of Peripheral Inflammation

Peripheral inflammation was induced under 3% isoflurane anesthesia. A 1% mixture of carrageenan in a physiological solution ($\sim$30 µl) was used to induce peripheral inflammation in mice[2], whereas 3% mixture of carrageenan with saline ($\sim$50 µl) was used in $\sim$P20 rats[3]. Carrageenan was injected subcutaneously to both hind paws. Mechanical paw withdrawal threshold (PWT; see SECTION 12.6, p. 81) or paw withdrawal latency (PWL; see SECTION 12.7, p. 82) to thermal stimuli was tested before carrageenan injection (on Day 0), and after on Day 1 ($\sim$24 h later), before spinal cord slices preparation and patch-clamp experiment. Naive animals were used as controls.

12.5.3. Model of Chronic Constriction Injury

CCI was performed in adult mice under 3% isoflurane anesthesia. Three loose ligatures were tied proximal to the trifurcation of the sciatic nerve. Mechanical withdrawal threshold (see SECTION 12.6, p. 81) was tested before (Day 0), and after CCI on Day 1 and 3, before spinal cord slices preparation and patch-clamp experiment. Naive animals were used as controls.

12.5.4. Burn injury model

The animal model of *partial thickness scalding-type burn injury* was used. Male Wistar rats (P21) were deeply anesthetized with 3% isoflurane. The absence of any nocifensive response was confirmed and under continuous anesthesia, both hind paws were immersed into 60 °C (Burn injury group) or 37 °C (Sham group) water up to the knee for 2 minutes. Continuous anesthesia (isoflurane, 3%) was maintained for the next 60 minutes. Then the laminectomy and spinal cord slices were prepared as described in SECTION 12.4, p. 80.

12.6. Behavioral Testing of Mechanical Withdrawal Threshold and Data Analysis

To perform behavioral tests, mice were placed on a stainless steel wire mesh under clear acrylic glass cages in a quiet room and allowed to acclimate for $\sim$1 hour. Paw withdrawal threshold (PWT) to tactile stimulation was tested manually in the morning hours (8:00 to 11:00 AM). Control PWT was tested in all groups on Day -3 and 0 before any treatment. We used both electronic von Frey apparatus (IITC Life Sciences, Model 2390 Series, USA) and series of 6 von Frey filaments with bending forces 0.16, 0.4, 1, 2, 4 and 10 g (Aesthesio™, DanMic Global, USA). The probe tip of electronic von Frey was applied 5 times to the plantar surface. The average

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[2] This approach was used in the study of mechanisms of disinhibition in the transgenic VGAT-ChR2-eYPF mice.

[3] This approach was used in the study of the effect of 20:4-NAPE on synaptic transmission under inflammatory conditions in young Wistar rats.

value from each hind paw was calculated and then averaged in the experimental group. Von Frey filaments were applied 5 times to the hind paw plantar surface. A quick flick or full paw withdrawal was considered a response. The averaged numbers of responses on each bending force were calculated for each hind paw and then averaged in the experimental group. All data are expressed as mean $\pm$ standard error of the mean (SEM). SigmaStat 3.5 software (SyStat, USA) was used for statistical analysis. Data from experiments with electronic von Frey apparatus were analyzed by Two Way ANOVA (treatment $\times$ time) followed by multiple comparison procedure versus Vehicle + PAC group and versus control Day 0 (Bonferroni post hoc test). Data from experiments with von Frey filaments were tested by non-parametric Friedman Repeated Measures ANOVA or by Kruskal-Wallis One Way ANOVA, both followed by Dunn's post hoc test to multiple comparisons versus control Day 0 or control treatment. The criterion for statistical significance was P < 0.05.

12.7. Behavioral Testing of Thermal Withdrawal Threshold and Data Analysis

The young Wistar rats used for the preparation of peripheral inflammation model were tested to thermal stimuli before and 24 hours after the model induction. Paw withdrawal latency (PWL) to radiant heat stimuli was measured for both hind paws using Plantar test apparatus 37370 (Ugo Basile, Italy). The tested rats were placed under the clear plastic cages on the glass plate of the apparatus. Animals were left to adapt to these condition at least for 20 min before the behavioral testing. The heat stimuli were applied to the plantar surface of each hind paw until the escape movement. This movement was automatically detected and measured by the Plantar test. The PWLs were measured four times for each paw. At least 5 min interval was between each measurement. The values obtained from each trial were averaged for each hind paw. The baseline PWLs were determined before any experimental procedure for all tested rats.

12.8. Patch-clamp Recording

Whole-cell patch-clamp recordings were made from visually identified superficial dorsal horn neurons using a differential interference contrast (DIC) microscope Zeiss Axio Examiner A.1 (Carl Zeiss Microscopy, Germany) equipped with infrared LED diode illumination and an infrared-sensitive camera Grasshopper 3 (Point Grey, Canada), connected to a standard personal computer. Patch pipettes were pulled from borosilicate glass tubing (Rückl Glass, Czech Republic) on Pipette Puller P-97 (Sutter Instruments, USA) and then filled with an intracellular solution for final resistance of 3.5–7.0 MΩ. The intracellular pipette solution contained (in mM): 125 gluconic acid lactone, 15 CsCl, 10 EGTA, 10 HEPES, 1 CaCl$_2$, 2 Mg$_2$ATP, 0.5 NaGTP and was adjusted to pH 7.2 with CsOH. Voltage-clamp recordings in the whole-cell configuration
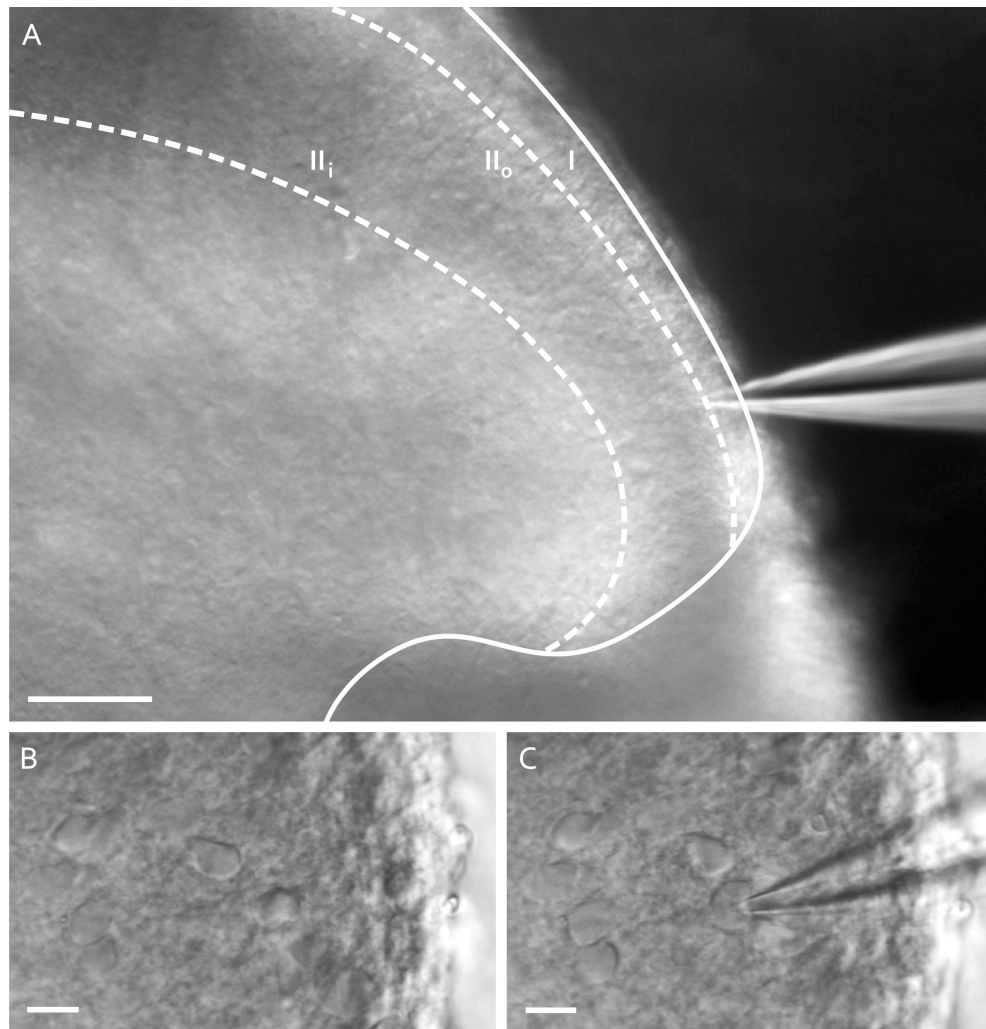
Figure 12.1.: **Whole-cell Patch-clamp recording from spinal neurons.** (**A**) The continuous line shows the dorsal horn of the spinal cord. The dotted line shows superficial laminae I and II. Lamina II is further divided into II$_o$ (*outer*) a II$_i$ (*inner*); Scale = 10 µm. In figures (**B**) and (**C**) is illustrated the formation of a so-called *"gigaohm seal"* between the microelectrode and the plasma membrane of the neuron; scale = 1 µm (Figure adapted from Adámek, 2014, Diploma Thesis).

were performed with an Axopatch 1D (Axon Instruments, USA) amplifier and Digidata 1440A digitizer (Molecular Devices, USA) at room temperature ($\sim$23 °C). Whole-cell recordings were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. The series resistance of neurons was routinely compensated by 80 % and was monitored during the whole experiment.

AMPA-mediated spontaneous or miniature excitatory postsynaptic currents (sEPSCs/ mEPSCs) were recorded from visually identified superficial dorsal horn neurons in laminae I and outer II, clamped at -70 mV in the presence of 10 µM bicuculline and 5 µM strychnine in the bath solution. For the recording of mEPSCs, bath solution contains also VGSCs blocker tetrodotoxin (0.5 µM).

GABA$_A$R- and/or GlyR-mediated sIPSC and light-evoked (le-IPSC) were routinely recorded using the same intracellular and recording solution at 0 mV. IPSCs were recorded in the presence of AMPA receptor antagonist CNQX (20 mM) and NMDA blocker AP5 (25 mM).

Software package pCLAMP 10.5 (Axon Instruments, USA) was used for data acquisition and for off-line analysis. Only sEPSC with an amplitude of 5 pA or greater (which corresponded to at least double the recording noise level) were included in the frequency and amplitude analysis.

12.8.1. Recording Protocol and Data Analysis Used in a Model of Paclitaxel-Induced Peripheral Neuropathy[4]

First, we studied the effect of the acute application of PAC (50 nM) on s/mEPSC. To study the role of TRPV1 in PAC-mediated modulation of synaptic transmission, specific TRPV1 antagonist SB366791 (10 µM) was used.

In the next set of experiments, the effect of paclitaxel (PAC; 50 nM) application on tachy-phylaxis of the second capsaicin response was studied as a change of mEPSCs frequency. Recording of mEPSCs began ∼4 min after whole-cell access when the recorded current had reached steady state. After recording of the control segment/basal activity (3 min), capsaicin (200 nM) was applied twice for 2 min with a 10 min interval in between the applications. In the control groups, only recording solution was perfused between the first and the second capsaicin application, while in the experimental groups PAC (50 nM) or PAC (50 nM) with other drugs (WMN, 500 nM; STAURO, 250 nM; LY, 20 µM) was co-applied. To study the role of TLR4 in paclitaxel mediated TRPV1 modulation, we used also another TLR4 agonist LPS 2 (µg/ml) and specific TLR4 antagonist, LPS-RS (2 µg/ml).

Data segments of 1–2 min duration were manually evaluated for each experimental condition. Data are expressed as mean ± standard error of the mean (SEM). The frequency of mEPSC is presented in absolute values (Hz) or is normalized as a percentage of the first capsaicin response (100 %). For statistical analysis, SigmaStat 3.5 software (SyStat, USA) was used. To find significant differences between groups with normal data distribution, One Way ANOVA followed by Bonferroni post hoc test (multiple comparison procedure versus control or all pairwise comparison procedure) was used. To find significant differences in one group during the treatment, we used One-Way repeated measures ANOVA. For comparison of standardized data with non-normal distribution, non-parametric Wilcoxon Signed Rank Test was used. The criterion for statistical significance was $P < 0.05$.

12.8.2. Recording Protocol Used to the Recording of Light-Evoked IPSC in Transgenic Mice

Transgenic male mice were used to the preparation of different pain models—including PIPN, carrageenan-induced peripheral inflammation, and CCI; nevertheless, the recording protocol used was the same.

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[4] Except for the experiments on transgenic mice in which IPSC were studied and different recording protocol was used (see Section 12.8.2, p. 84).

Following the recording of sEPSC at -70 mV, the inhibitory/excitatory neurons were distinguished by application of 500 ms long blue light (470 nM) photo-stimulation (for a detailed description, please see results, SECTION 13.2.2 (p. 106). Only excitatory neurons were used for the next analysis.

Light-evoked IPSC (le-IPSC) were recorded as a series of ten 5 ms long photo-stimulations with a frequency 0.1 Hz. These ten responses were averaged for each cell/each experimental condition. Amplitudes, an area under the curve, rise time and decay time were analyzed. To a more detailed description of le-IPSC, the time constant *Tau* was fitted. le-IPSCs was best fitted with standard bi-exponential function with the Chebyshev method using Clampfit 10.5. software. All values in results are expressed as a mean with SEM. To evaluate statistically significant differences between groups, One-Way repeated measures ANOVA followed by Bonferroni post hoc test were used. The criterion for statistical significance was $P < 0.05$.

12.8.3. Recording Protocol and Data Analysis Used in a Burn Injury Model

In this model was studied the effect of $Na_v1.7$ antagonist ProTxII on sEPSC frequency. Basal activity of each recorded neuron was recorded first, and then ProTxII (10 nM in 0.1% BSA) was applied for 5 min. Data segments of 3 min duration were analyzed for both conditions. At the end of the recording protocol, neurons with capsaicin-sensitive input were identified by an increase of sEPSC frequency following capsaicin (200 nM) application. Capsaicin responses were analyzed in a segment of 20 s duration. Mean frequency and SEM were calculated. Statistical significance was tested using paired t-test and t-test with Bonferroni correction for multiple comparisons.

12.8.4. Recording Protocol and Data Analysis Used in the Study of Peripheral Inflammation With a Focus on the Role of CB_1 Receptors

The aim of this experiment was to show the effect of CB_1 antagonists PF514273 on 20:4-NAPE-induced inhibition of sEPSCs frequency under the inflammatory conditions.

After recording of the control segment/basal activity (4 min), 6 min antagonist pretreatment with PF514273 preceded the 4 min co-application of PF514273 with anandamide precursor 20:4-NAPE. Neurons with TRPV1 expressing/capsaicin-sensitive primary afferents were identified by an increase of sEPSCs frequency after capsaicin (200 nM) application at the end of the recording protocol.

To evaluate statistically significant differences, One-Way repeated measures ANOVA followed by the Student-Newman-Keuls test was used. The criterion for statistical significance was $P < 0.05$.

12.9. Immunohistochemical Analysis of Akt Kinase Phosphorylation After Acute Paclitaxel Treatment

Wistar rats (6 weeks old, 180–200 g) were randomly distributed in three experimental groups: Control (CTRL group, n = 5); paclitaxel (PAC group, n = 5); wortmannin + paclitaxel (WMN + PAC, n = 5). Control animals were treated for 1 hour with an equivalent volume of the vehicle only (1:1 Kolliphor EL:ethanol) diluted in saline to reach the same concentration of vehicle as in paclitaxel solution. Animals in the PAC group received 8 mg/kg *i.p.* Paclitaxel Mylan for 1 hour. Animals in WMN + PAC group were pretreated with wortmannin (0.6 mg/kg) 1 hour before paclitaxel administration. Animals were deeply anesthetized with a combination of ketamine (100 mg/kg, Narketan, Zentiva) and xylazine (25 mg/kg, Xylapan, Zentiva) 1 hour after paclitaxel/vehicle treatment and perfused intracardially with saline followed by ice-cold 4% paraformaldehyde. The L5 DRGs were removed and post-fixed in 4% paraformaldehyde at 4 °C for 2 hours, cryoprotected with 30% sucrose overnight, and cut in cryostat Leica CM3000 to 16 µm thick slices.

These sections were then processed for pAkt immunohistochemistry. Briefly, sections were washed 3× for 10 min in phosphate-buffer solution (PBS), blocked with 3% normal donkey serum (NDS) for 30 min at room temperature and incubated overnight at 4 °C with rabbit anti-pAkt (Ser473; 1:200; Cell Signaling Technology, No. 4060S, Netherlands) primary antibody in 1% NDS with 0.3% Triton X-100. After washing in 1% NDS (3× for 10 min), the sections were exposed to a donkey anti-rabbit Cy2-conjugated secondary antibody (1:400, Jackson Immuno Research Lab. Inc, USA) for 2 hours. For visualization of the cell nucleus, incubation in bisbenzimide (Hoechst 33342, Sigma-Aldrich) for 3 min was used.

All sections were visualized and captured using a fluorescence microscope equipped with a digital camera system (Olympus BX53). The region of interest (ROI) was outlined for each DRG section representing only regions of the sensory ganglia containing neuronal cell bodies (excluding nerve fibers). Area analysis was performed only in these ROIs. Area of pAkt-immunoreactivity (IR) in this region was measured using ImageJ software (NIH, USA) threshold function. To set proper threshold value we obtained intensity values of multiple pAkt-IR cell bodies. IR/ROI ratios were calculated and expressed as a percentage (IR %). To analyze a number of pAkt-IR DRG cells all cell bodies in DRG sections were manually outlined and intensity and area of individual cell bodies were measured using ImageJ software. Cell bodies were divided into pAkt-IR and pAkt-non-IR groups based on their intensity. Only cells with visible nucleus were included into the analysis. All data are represented as mean ± SEM. The difference between the groups was compared using a paired t-test or One Way ANOVA followed by Bonferroni post hoc test in SigmaStat software. The criterion for statistical significance was $P < 0.05$.

12.10. Immunohistochemical Analysis of c-Fos Protein Expression in the *in vitro* Model of PIPN

Acute spinal cord slices for this immunohistochemical experiment were prepared from juvenile male Wistar rats (P21) by the same procedure as was described previously in SECTION 12.4 (p. 80). The only difference is that 350 μm thick acute transverse slices were cut. Altogether, we used 15 animals in this experiment.

Spinal cord slices from each animal were randomly assigned to groups with different incubation protocol: (I.) A control group (CTRL group) of slices was incubated with vehicle (DMSO, 70 min). (II.) Paclitaxel group (PAC 100 nM, 60 min); (III.) TRPV1 antagonist pretreatment (SB366791 or AMG9810, 10 μM, 10 min) + Paclitaxel (PAC 100 nM, 60 min)—SB + PAC or AMG + PAC groups; (IV.) TRPV1 antagonists only group (SB or AMG group, 10 μM, 70 min). The effect of SB366791 or AMG9810 was tested in a separate series of experiments.

Incubation solution was saturated with carbogen (5 % CO_2 and 95 % O_2) during the whole experiment. Slices were fixed in 4% paraformaldehyde solution overnight, cryoprotected with 30% sucrose solution and cut in cryostat Leica CM3000 to 16 μm thick sections from the middle part of the slices. 15–20 sections were performed from each slice. These sections were then immunohistochemically processed for detection of c-Fos protein expression.

Immunostaining was performed by the streptavidin-biotin-peroxidase complex method (SABC). First, sections were blocked with 3% NDS, incubated overnight at 4 °C with the anti-c-Fos antibody (rabbit, 1:2000; Santa Cruz, USA) in 1% NDS with 0.3% Triton X-100. For SABC staining, the sections were incubated for 2 hours with biotinylated secondary antibody (1:400) and 2 hours with peroxidase-conjugated streptavidin (1:400, Jackson Immuno Research, USA). The reaction product was visualized with 1.85 mM DAB (3,3'-diaminobenzidine)/0.003 % hydrogen peroxide in PBS for 2–5 min (Sigma-Aldrich, USA).

Finally, sections were photographed and analyzed using ImageJ software. Laminae I/II of the spinal cord dorsal horn were manually outlined and measured (in pixels). Area and the number of immunoreactive neuronal nuclei for c-Fos in this region were counted. For every section was calculated the number of labeled neurons per measured area ratio (Cells/area (c/a) ratio). c/a ratios were counted and averaged from sections for each slice. From 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 in SigmaStat software. The criterion for statistical significance was $P < 0.05$.

12.11. Anandamide Release Experiment

Spinal cord slices from five juvenile rats (P21) were prepared in the same way as for electrophysiological patch-clamp experiment (see SECTION 12.4, p. 80) 18 acute spinal cord slices were used

for each of five experiments. Slices were put into a plastic tube filled with recording solution, saturated with carbogen (5 % CO_2 and 95 % O_2) during the incubation period. Slices were during experiment incubated for 10 min in recording solution with a different concentration of anandamide precursor 20:4-NAPE. After each 10 min long incubation, the whole volume of the solution was extracted from the tube, immediately frozen (for latter mass spectrometry experiment) and the solution was immediately replaced and the slices were incubated with another solution sample for next 10 min.

In total, eight solution samples were taken after incubation of slices: (I.) Recording solution only; (II.) Recording solution only; (III.) 20:4-NAPE (20 µM); (IV.) Recording solution only; (V.) 20:4-NAPE (100 µM); (VI.) Recording solution only; (VII.) 20:4-NAPE (200 µM); (VIII.) Recording solution only.

Additional samples with only 20:4-NAPE (20, 100, and 200 µM) were also prepared and analyzed by mass spectrometry.

All of the collected samples were analyzed for the presence of anandamide with mass spectrometry in the Department of Analysis of Biologically Important Compound (Institute of Physiology of the Czech Academy of Sciences) by the team of Prof. Ing. Ivan Mikšík, DrSc. For a detailed description of mass spectrometry, please see methods in Nerandžič *et al.* (2018).

13. Results

13.1. The Role of TLR4 and TRPV1 Signaling in the Paclitaxel-Induced Peripheral Neuropathy

The increasing body of evidence suggests that TLR4 receptor play an important role in the modulation of different inflammatory pain states. In following experiments, we focused on the mechanism if, and subsequently how, PAC modulates nociceptive synaptic transmission, and which role play TLR4 and TRPV1 receptors in this process.

13.1.1. Acute Paclitaxel Application Increases mEPSCs, but not sEPSCs or eEPSCs Frequency in Spinal Cord Neurons[1]

To investigate the mechanisms of the paclitaxel (PAC)-induced modulation of synaptic transmission, we performed whole-cell patch-clamp recording of AMPA mediated miniature- (mEPSCs), spontaneous- (sEPSCs) and evoked excitatory postsynaptic currents (eEPSCs) recorded in the superficial dorsal horn neurons in rat spinal cord slices.

The mEPSCs activity was recorded in two groups of neurons. In the first one, only PAC was applied, while in the second group pretreatment with specific TRPV1 antagonist SB366791 was used before and during the PAC application. As shown in the Fig. 13.1. A, application of PAC (50 nM; 10 min) increased significantly the average frequency of mEPSCs to 140.7 ± 11.1 % (n = 14; P < 0.001) relative to the control value before the treatment (100 %; Fig. 13.1.A and C). This increase was present already at 6 min after the beginning and was sustained until the end of the application at 10 min. In the second group of neurons, pretreatment with SB366791 (10 µM; 4 min) did not change the average mEPSCs frequency (101.5 ± 9.1 %; n = 10; P > 0.05; Fig. 13.1.B and C). Following co-application of TRPV1 inhibitor SB366791 (10 µM) and PAC (50 nM), the effect of PAC was blocked. The average mEPSC frequency did not differ from the control values (90.0 ± 6.2 %; n = 10; P > 0.05; Fig. 13.1.B and C). These results demonstrate that PAC-induced increase of the mEPSCs frequency is dependent on functional/unblocked TRPV1 receptors.

The mean amplitude of the mEPSCs was not significantly changed in any of tested group. Only for illustration, in the first group of neurons, it was 20.9 ± 2.4 pA under the control

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[1] **Author contribution** to presented data adapted from the study Li *et al.* (2015).: P. Adámek (P.A.) conducted the recording and the subsequent data analysis of presented sEPSCs. P. Mrózková conducted the recording and data analysis of mEPSC and eEPSCs. P.A. participated in the writing of the paper.
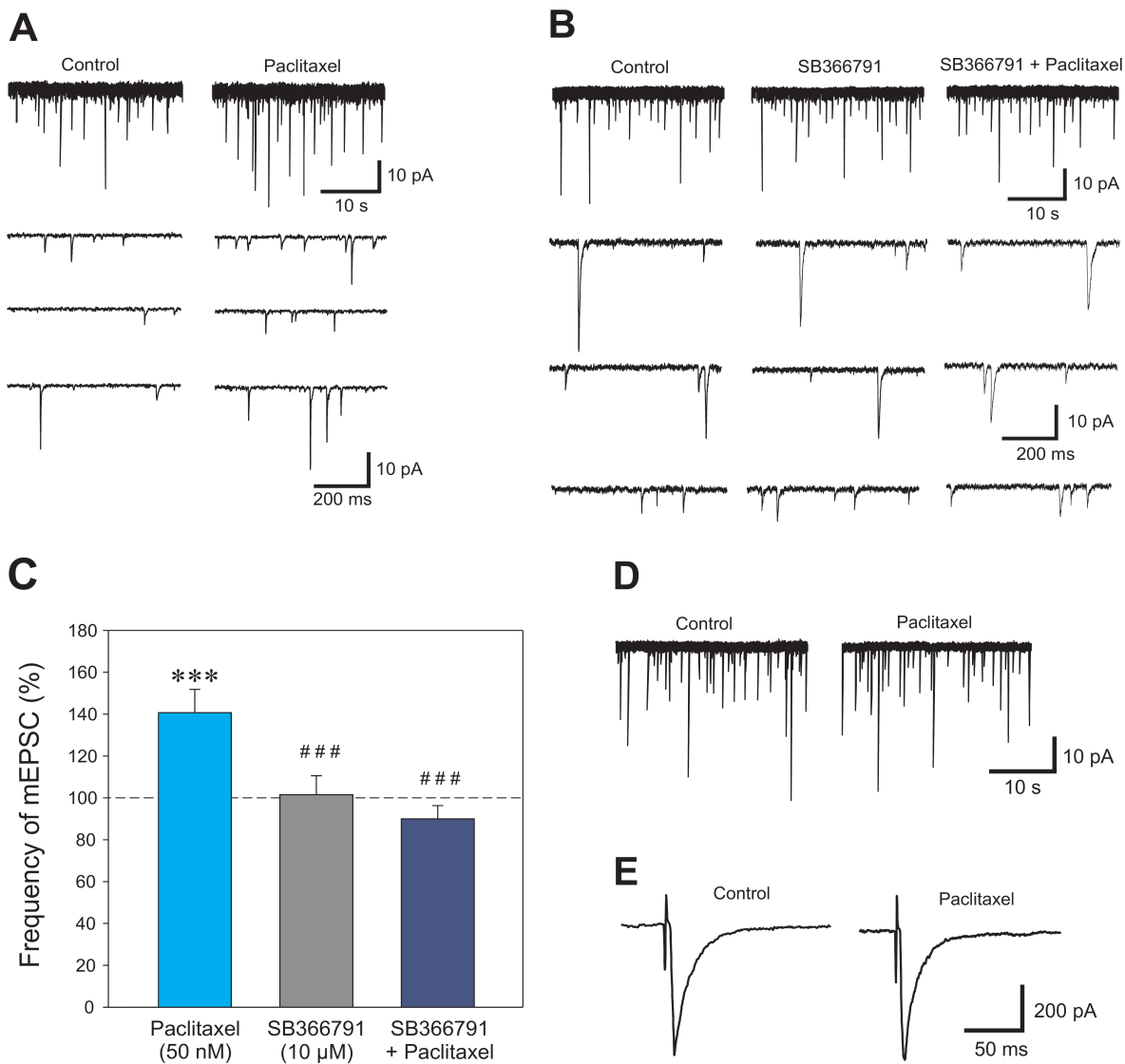
Figure 13.1.: **Paclitaxel application increased the frequency of mEPSCs in superficial dorsal horn neurons in rat spinal cord slice.** (**A**) Native recording of mEPSC activity before and after paclitaxel (PAC; 50 nM) application. (**B**) The application of TRPV1 antagonist SB366791 (10 µM) did not change the mEPSC frequency but prevented its increase during SB366791 (10 µM) + PAC (50 nM) co-application. (**C**) Averaged responses demonstrate that PAC treatment induced significant increase of mEPSCs frequency compared with the baseline (control; 100%) value (140.7 ± 11.1 %; n = 14). This increase was prevented by the TRPV1 antagonist (SB366791 + PAC) treatment, while the antagonist alone did not have any effect (SB366791; n = 10). (**D**) PAC application did not change the frequency and amplitude of the sEPSCs as well as the amplitude of the dorsal root stimulation evoked eEPSCs (**E**). One-way ANOVA followed by Student-Neuman-Keuls test was used for statistical analyses; ***$P < 0.001$ versus control values; ###$P < 0.001$ versus paclitaxel.

conditions and did not change significantly during PAC application (17.3 ± 2.0 pA; $P > 0.05$). Similarly, in the second group of neurons, the control mEPSCs amplitude was 21.5 ± 3.4 pA and did not change significantly ($P > 0.05$) during the SB366791 or SB366791 + PAC co-application (21.7 ± 4.3 pA or 19.2 ± 3.5 pA, respectively. In the first group of recorded neurons without the TRPV1 antagonist treatment, 12 out of the 14 neurons responded to capsaicin application at the end of the experiment, suggesting TRPV1-positive inputs from primary sensory nociceptors on these neurons, and hence, their involvement in nociceptive signaling.

Surprisingly, we did not observe any significant changes in the average frequency of the sEPSCs or in the amplitude of the eEPSCs after the acute PAC (50 nM) treatment. The average frequency of sEPSCs after PAC application was 104.4 ± 9.0 % (n = 14; $P > 0.05$), when compare to the control value before the PAC application (100 %; FIG. 13.1.D). However, the responses induced by PAC were not homogenous across the population of tested neurons; 6 of the 14 neurons showed an increase of the sEPSC frequency (>10 %; 135.1 ± 10.8 %; $P < 0.05$), 6 neurons showed a decrease of the sEPSC frequency (>10 %; 74.1 ± 2.8 %; $P < 0.001$) and in 2 neurons the frequency did not change (103.3 ± 2.5 %). Mean amplitude of the control sEPSCs was 16.9 ± 1.0 pA and did not change significantly during the PAC application (16.7 ± 0.8 pA; $P > 0.05$).

Similarly, we did not find any significant changes in the amplitudes of the eEPSCs evoked by dorsal root stimulation. PAC did not change the average amplitude being 104.7 ± 5.2 % (n = 9; $P > 0.05$), when compare with the pre-application control values (FIG. 13.1.E).

These results indicate that acute PAC treatment may increase via TRPV1-dependent mechanism action potential-independent spontaneous release of glutamate from presynaptic endings in the dorsal horn (recorded as mEPSCs), whereas, acute PAC did not affect action potential-dependent eEPSCs and sEPSCs when action potential propagation was not blocked by TTX. However, this may be changed following chronic PAC treatment, when sEPSCs are also increased (Li *et al.*, 2015a).

13.1.2. Acute Paclitaxel Application Enhances Spinal TRPV1 Responses via TLR4[2]

The possibility that PAC increases mEPSC frequency by an effect mediated by TLR4 was tested in the dorsal horn neurons, in slices prepared from adult mouse. As shown in the top line of the representative recordings under the control situation (FIG. 13.2.A), an initial application of capsaicin (200 nM) evoked an increase in mEPSCs frequency (9.9 ± 1.7 Hz). Ten minutes later, the second response to capsaicin was notably reduced, compared with the first, because of tachyphylaxis (FIG. 13.2.A and D). FIG. 13.2.B (middle trace) shows that PAC (50 nM), when applied alone after an initial application of capsaicin, evokes an increase in mEPSCs, but

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[2] **Author contribution** to presented data adapted from the study Li *et al.* (2015). P. Adámek (P.A.) conducted all of the recordings and the subsequent data analysis of mEPSCs presented in this section. P.A. participated in the writing of the paper.
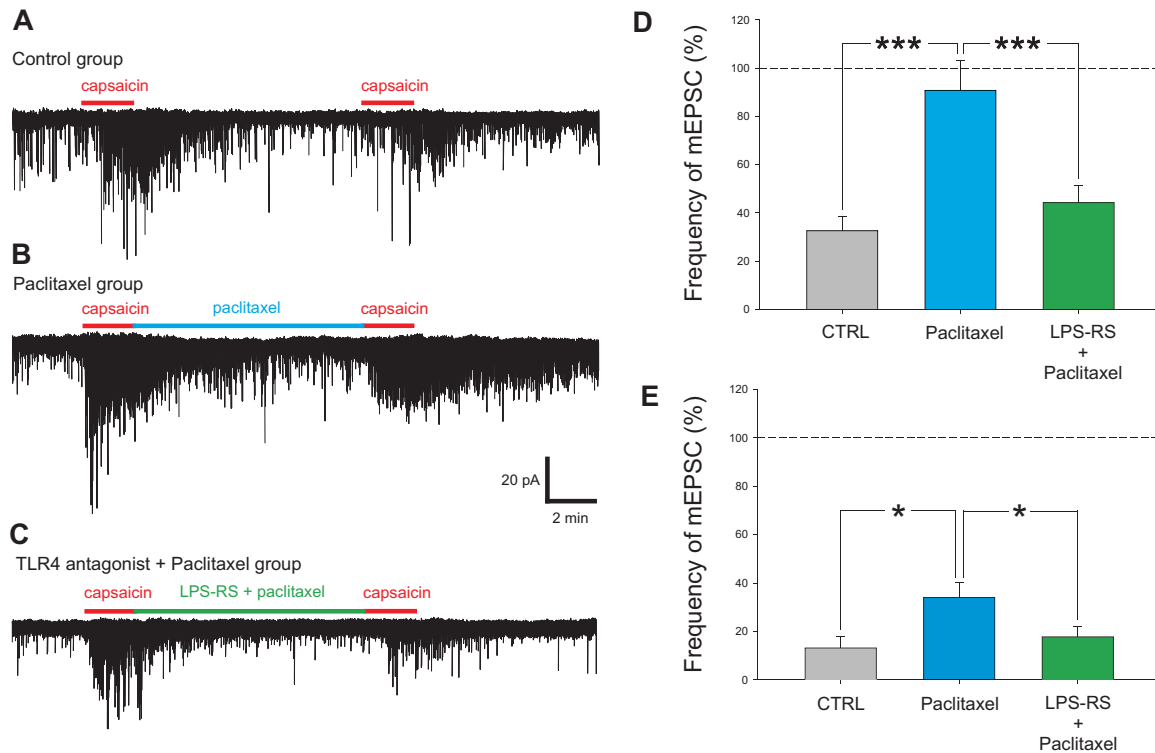
Figure 13.2.: **Acute paclitaxel treatment prevented the tachyphylaxis of second capsaicin response via a TLR4-dependent mechanism.** Representative traces showing that capsaicin (200 nM) increases mEPSCs in spinal neurons and that the second response was notably reduced compared with the first one in control, vehicle-treated mice (**A**). Acute application of PAC (50 nM) for 10 min before the second capsaicin application prevented the decrease of the second response, i.e., reduced the tachyphylaxis of the second capsaicin response (**B**). Coapplication of PAC with the TLR4 antagonist LPS-RS (2 μg/ml) prevented the effect of acute PAC on the second capsaicin response, with the result being tachyphylaxis, as seen in the control group (**C**). In (**D**), the mean normalized responses are shown for each group, where the responses to the second capsaicin application are expressed as a percentage of the first capsaicin application (100 %). The mean increase in mEPSCs frequency 5 min after the first capsaicin application was prolonged in the presence of PAC (**E**). One-Way ANOVA followed by Student-Newman-Keuls test was used for statistical analyses; ***$P < 0.001$; *$P < 0.05$ versus PAC group.

also prevents the tachyphylaxis, i.e., decrease in response to the second capsaicin application. Coapplication of TLR4 antagonist LPS-RS with PAC (FIG. 13.2.C) prevented both the acute increase in mEPSC frequency induced by PAC and the salvage of the second capsaicin response.

In FIG. 13.2.D, the mean normalized responses are shown for each group, where the responses to the second capsaicin application are expressed as a percentage of the first application (100 %). The second response to capsaicin averaged 32.6 ± 6.0 % of the first in the control group (CTRL, $n = 8$). In contrast, the second response to capsaicin applied after PAC was 90.8 ± 12.3 % of the first response (Paclitaxel, $n = 10$). Finally, the second response to capsaicin averaged 44.2 ± 7.3 % of the first in the LPS-RS + Paclitaxel group (right bar, $n = 12$).

The increase in mEPSC frequency 5 min after the first capsaicin application was protracted in the presence of PAC (Fig. 13.2.E). The mEPSCs frequency at the 5–6 min interval in the paclitaxel group (34.0 ± 6.1 % of the capsaicin response, n = 10) was significantly higher compared with the frequency in the control (13.1 ± 4.6 %; n = 8) and the LPS-RS + PAC (17.7 ± 4.4 %; n = 12) groups.

In these experiments, the amplitudes of recorded mEPSCs were not significantly different between the groups. After normalization to the value at the beginning of the recording (control segment before capsaicin application; 100 %), there were no significant changes detected in the average mEPSCs amplitude during the course of the recording in all three experimental groups (Li *et al.*, 2015a).

13.1.3. Acute *in vitro* and Systemic *in vivo* Paclitaxel Treatments Enhanced Responses of Dorsal Horn Neurons to Repeated Capsaicin Applications

Our previous experiments have shown that an acute PAC application on spinal cord slices increased TRPV1-mediated capsaicin responses in dorsal horn neurons via the TLR4 dependent mechanism (Li *et al.*, 2015a). The first aim of the subsequent experiments was to study the modulation of TRPV1 receptors tachyphylaxis, recorded as changes in mEPSCs frequency evoked by capsaicin in the superficial DH neurons after different treatments: (I.) an acute application of PAC (50 nM, PAC acute); (II.) an acute application of LPS, another TLR4 agonist (10 μg/ml, LPS acute); (III.) incubation of slices with PAC (∼2 h; 50 nM, PAC incubation); (IV.) and 1 or (V.) 8 days after a single systemic PAC injection (8 mg/kg, *i.p.*, PAC Day 1 and PAC Day 8).

Results summarized in Fig. 13.3.A show that the control basal mEPSCs frequency was low and did not differ in the first three groups, where the slices were without any treatment: Naive control 0.73 ± 0.20 Hz (n = 9); LPS acute 0.73 ± 0.23 Hz (n = 8) and PAC acute 1.01 ± 0.19 Hz (n = 11). However, the basal mEPSCs frequency increased, although not significantly, after the PAC incubation (2.02 ± 0.33 Hz; n = 8) as well as in PAC Day 1 group (2.45 ± 0.78 Hz; n = 9). Basal mEPSCs frequency increased significantly only in PAC Day 8 group (3.11 ± 0.60 Hz; n = 12; $P < 0.05$; Fig. 13.3.A). Representative recordings from the Naive control group and the PAC Day 8 group are shown in Fig. 13.3.B.

Next, we evaluated how different PAC (or LPS) treatments modulate sensitivity to the first capsaicin (0.2 μM) application, measured as a change in frequency of the evoked mEPSCs activity. In the groups without any prior treatment (Naive control, LPS acute, PAC acute), the capsaicin application evoked a similar response (Fig. 13.3.C). In the Naive control 11.78 ± 2.40 Hz (n = 9), in the LPS acute group 13.01 ± 2.08 Hz (n = 8), and in the PAC acute group 7.78 ± 1.64 Hz (n = 11). Incubation in PAC for ∼2 h did not change the first capsaicin response (9.29 ± 1.98 Hz; n = 8). However, after the PAC *i.p.* treatment, there was a robust increase in PAC Day 1 (22.23 ± 3.91 Hz; n = 9) and in PAC Day 8 (26.24 ± 5.48 Hz; n = 12; $P < 0.05$).
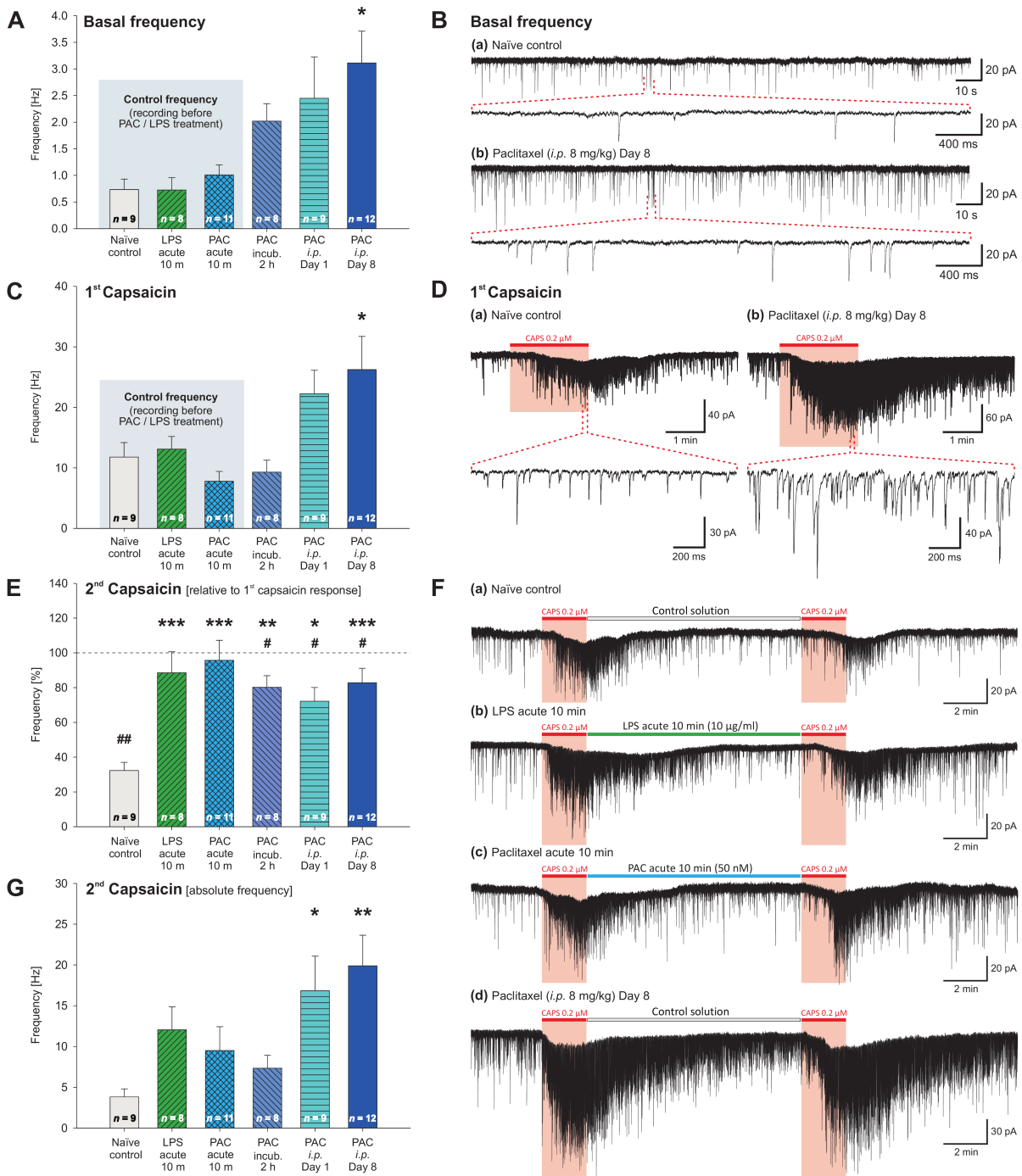
Figure 13.3.: **Effects of different paclitaxel (PAC) treatments on the mEPSCs frequency after the capsaicin (CAPS) application**. (**A**) The basal mEPSCs frequency was increased significantly in neurons recorded eight days after the single PAC treatment *i.p.* (**B**) Representative recordings of basal mEPSC in the Naïve control (**a**) and eight days after the systemic PAC treatment (**b**). (**C**) Responses to the first CAPS application were increased in neurons eight days after the PAC *i.p.* treatment (*P < 0.05). (**D**) Representative recordings of responses to the first CAPS application in the Naïve control (**a**) and PAC Day 8 (**b**) neurons. (**E**) In the Naïve control group, the second CAPS response was distinctly reduced compared with the first one (##P < 0.01). Tachyphylaxis of the second CAPS response was dramatically reduced after the LPS or various PAC treatments versus the Naïve control (*P < 0.05; **P < 0.01; ***P < 0.001). The second CAPS response was different from the first one in the groups: PAC incub. 2h, PAC Day 1 and PAC Day 8 (#P < 0.05). (**F**) Representative responses to repeated CAPS application with different treatments. (**G**) The absolute frequencies of the second CAPS response on PAC Day 1 and PAC Day 8 were significantly enhanced (*P < 0.05; **P < 0.01). The One-Way ANOVA followed by a multiple comparison procedure versus the Naïve control group (the Bonferroni post hoc test) was used. For comparison of differences between the first and the second CAPS in each group (**E**), the nonparametric Wilcoxon signed rank test was used.

Examples of the responses to the first capsaicin application in Naive controls and on Day 8 after the PAC *i.p.* are shown in Fig. 13.3.D.

To account for the differences between the individual neurons, the responses to the second capsaicin are given as relative values to the first capsaicin responses (100 %) in each group (Fig. 13.3.E). In the control Naive group, there was a significant tachyphylaxis of the second capsaicin response (32.2 ± 5.3 %; n = 9; P < 0.01) in comparison with the first response (Fig. 13.3.E and Fa). Acute applications of TLR4 agonist LPS (Fig. 13.3.Fb) or PAC (Fig. 13.3.Fc) ten minutes before the second capsaicin application significantly increased the frequency of mEPSCs and reduced the tachyphylaxis of the second capsaicin responses following PAC (88.6 ± 12.0 %; n = 8; P < 0.001) and LPS treatment (95.7 ± 11.5 %; n = 11; P < 0.001) compared to the Naive control. The tachyphylaxis of the second capsaicin response was also significantly diminished after the 2 h PAC incubation (80.2 ± 6.7 %; n = 8; P < 0.01), on PAC Day 1 (72.2 ± 7.9 %; n = 9; P < 0.05) and on PAC Day 8 (82.8 ± 8.3 %; n = 12; P < 0.001; Fig. 13.3.Fd). The absolute frequencies of the second capsaicin responses (Fig. 13.3.G) were higher in all the experimental groups, but the differences against the Naive control (3.8 ± 1.0 Hz; n = 9) were significant only on PAC Day 1 (16.9 ± 4.2 Hz; n = 9; P < 0.05) and on PAC Day 8 (19.9 ± 3.8 Hz; n = 12; P < 0.01).

The mean amplitudes of the basal mEPSCs did not differ between the groups: Naive control 14.9 ± 1.6 pA, LPS acute 17.0 ± 2.0 pA, PAC acute 12.7 ± 0.8 pA, PAC incubation 16.0 ± 2.1 pA, PAC Day 1 19.2 ± 2.3 pA, and PAC Day 8 18.5 ± 1.4 pA. Amplitudes did not change significantly during the following treatment with first and/or second capsaicin in any of the experimental groups. Only for illustration, in the Naive control group, the mean basal amplitude was 14.9 ± 1.6 pA, 16.6 ± 0.6 pA after first, and 15.3 ± 1.2 pA after second capsaicin (n = 9); in the PAC Day 8 group, the mean amplitude was 18.5 ± 1.4 pA, 21.6 ± 1.3 pA after first, and 20.6 ± 1.7 pA after second capsaicin (n = 12). The representative recordings after the capsaicin application in Fig. 13.3.D and Fig. 13.3.F suggest the presence of a mEPSCs amplitude increase. However, this is only a graphical artifact mainly due to the *superposition* of individual mEPSC rather than a change in the individual mEPSC sizes. The superposition of mEPSCs is evident in the detail of the representative recording (Fig. 13.3.Db).

These results show that the activation of TLR4 receptors by acute applications of LPS, paclitaxel and by ∼2 h incubation in PAC solution all produce the decrease in the degree of tachyphylaxis of the second capsaicin response. Similarly, a single *i.p.* administration of PAC also induces robust changes in the tachyphylaxis of capsaicin response in slices taken from animals on Day 1 and on Day 8 after the single PAC treatment. Moreover, *in vivo* PAC treatment also produce increased first capsaicin response.

13.1.4. Co-Application of Wortmannin, LY-294002 and Staurosporine Prevented Acute Paclitaxel-Induced Reduction of the Second Capsaicin Response Tachyphylaxis in Naive Mice

To identify possible pathways involved in the reduction of the second capsaicin response tachyphylaxis after the acute PAC treatment, we considered modulating functions of several kinases such as PI3K, PKC, PKA, CaMKII, which are important for the TRPV1 receptor function. The potent irreversible PI3K antagonist wortmannin (WMN) and the specific PI3K inhibitor LY-294002 (LY) were used to inhibit PI3K signaling. Staurosporine (STAURO), a broad-spectrum protein kinase inhibitor, was used for the inhibition of serine/threonine kinases (such as PKC, PKA, CaMKII), involved in the TRPV1 modulation (Bonnington & McNaughton, 2003; Meents *et al.*, 2010; Wilkinson & Hallam, 1994).

Firstly, we tested the effects of acute WMN (500 nM), LY (20 µM) and STAURO (250 nM) applications on the second capsaicin response (Fig. 13.4.A). Individual applications of these antagonists 10 min before the second capsaicin did not change significantly the extent of tachyphylaxis (WMN: 39.5 ± 6.0 %, n = 9; LY: 29.1 ± 4.4 %, n = 7; STAURO: 39.6 ± 4.8 %, n = 9), when compared with the Naive control group (32.3 ± 4.7 %; n = 10). The second capsaicin responses were significantly lower than the first capsaicin responses in all of these experimental groups (Fig. 13.4.A; $P < 0.05$) similar to the Naive control group.

Individual acute PAC applications induced a significant reduction of the second capsaicin response tachyphylaxis in comparison with the control (Fig. 13.4.Ba). The second capsaicin response was comparable with the first one (Fig. 13.4.Bb). Co-applications of WMN, LY, and STAURO with PAC significantly abolished the PAC-induced tachyphylaxis reduction ($P < 0.001$). The relative size of the second capsaicin responses was also significantly reduced in comparison with the first responses (Fig. 13.4.A): the WMN + PAC group 37.7 ± 4.4 % (n = 10, $P < 0.01$), the LY + PAC group 36.9 ± 2.9 % (n = 8, $P < 0.01$) and the STAURO + PAC group 46.2 ± 7.0 % (n = 10, $P < 0.01$). Representative responses for each group are shown in Fig. 13.4.B(c-e).

An analysis of the mEPSCs did not reveal any significant changes in the mean amplitude during the treatment (control, first capsaicin, second capsaicin) in any of the tested groups compared to the Naive control group.

These results suggest that PAC-induced short-term alterations in the properties of TRPV1 receptors, recorded as changes in the mEPSCs frequency and manifested as reduced tachyphylaxis of the second CAPS response, are at least partially dependent on PI3K and serine/threonine protein kinases (PKC, PKA, CaMKII) signaling.
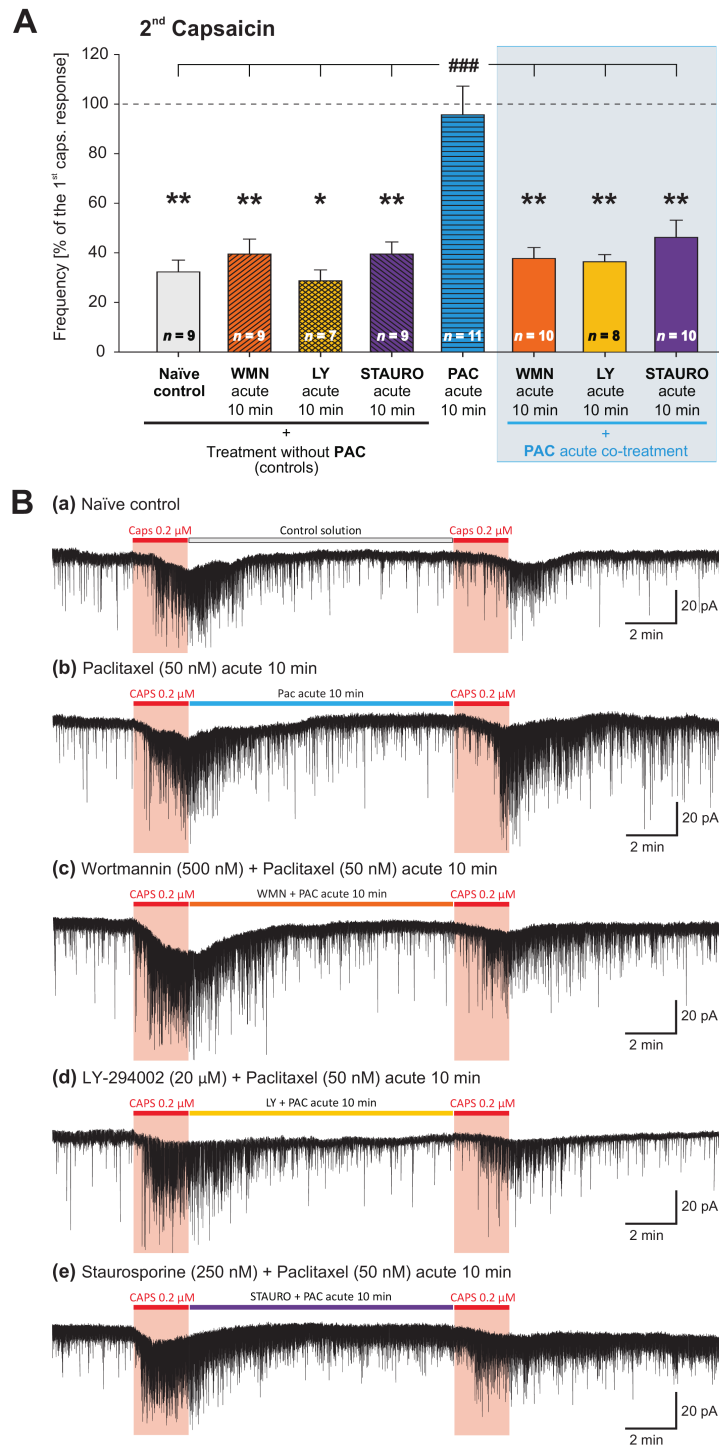
Figure 13.4.: **The acute application of wortmannin (WMN), LY-294002 (LY), and staurosporine (STAURO) significantly inhibits the PAC-induced reduction of the second capsaicin (CAPS) response tachyphylaxis.** (**A**) Neurons after acute application of WMN (500 nM), LY (20 µM), or STAURO (250 nM) had similar tachyphylaxis of the second CAPS response as those in the Naïve control (*P < 0.05; **P < 0.01, compared to the normalized first CAPS response). PAC (50 nM) induced a significant reduction of tachyphylaxis and the second CAPS response was therefore comparable with the first one. Co-application of PAC with WMN, LY, and STAURO significantly reduced the effect of PAC on tachyphylaxis ([###]P < 0.001) and the second CAPS responses were also significantly different from the first one (**P < 0.01). (**B**) Representative recordings of CAPS responses with different treatments. The One-Way ANOVA followed by a multiple comparison procedure versus the PAC acute group (the Bonferroni post hoc test) was used. For comparison of the first and the second CAPS in each group, the nonparametric Wilcoxon signed rank test was used.
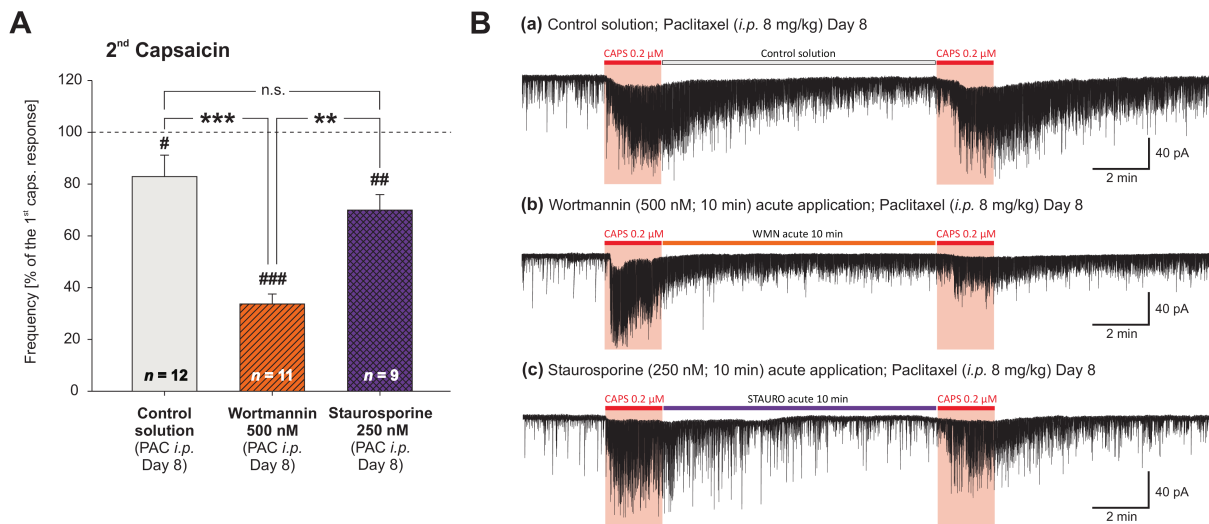
Figure 13.5.: **Effects of acute wortmannin (WMN) and staurosporine (STAURO) applications on capsaicin (CAPS) response eight days after the paclitaxel (PAC) *i.p.* treatment.** (**A, B**) Tachyphylaxis of the second CAPS response was dramatically reduced on Day 8 after the PAC i.p. treatment, while it was still different from the first CAPS (100 %; [#]P < 0.05). The acute WMN treatment before the second CAPS application significantly reduced the effect of PAC and enhanced tachyphylaxis (***P < 0.001) leaving the second CAPS different from the first CAPS (100 %; [###]P < 0.001). The acute STAURO application had a smaller effect in comparison with the WMN group (**P < 0.01) and the second CAPS response was different only from the first CAPS ([##]P < 0.01). The One-Way ANOVA followed by a multiple comparison procedure versus the control group (the Bonferroni post hoc test) was used. For comparison of the first and the second CAPS in each group, the nonparametric Wilcoxon signed rank test was used.

13.1.5. Acute Wortmannin Application Reduced the Inhibitory Effect of Paclitaxel on the Second Capsaicin Response Tachyphylaxis, Eight Days After a Single *in vivo* Paclitaxel Treatment

Here we tested if an acute application of WMN or STAURO can reverse the reduction of tachyphylaxis of the second capsaicin response present in spinal cord dorsal horn neurons recorded in slices taken from mice eight days after single PAC treatment (8 mg/kg; *i.p.*).

Acute WMN (500 nM) application 10 min before the second capsaicin dramatically enhanced tachyphylaxis of the response (33.7 ± 3.9 % of the first capsaicin; P < 0.001), as is evident from FIG. 13.5.A, and Bb. This was in contrast to the response recorded without any further acute treatment (82.8 ± 8.3 %; FIG. 13.5.A, and Ba). After an acute STAURO (250 nM) application 10 min before the second capsaicin, the response was 69.9 ± 6.0 %. This was significantly different from the first response (P < 0.001), but it did not differ significantly from the control without any further acute application (FIG. 13.5.A, and Ba, c). In comparison with the WMN effect, the acute application of STAURO had a significantly smaller effect on the relative size of the second capsaicin response (P < 0.01; FIG. 13.5.A, and Bc).

We did not observe any changes in the mean amplitude of the mEPSCs during the experiment (basal activity, first capsaicin, second capsaicin, respectively). Only for illustration, in the control solution group, it was 18.5 ± 1.4 pA, 21.6 ± 1.3 pA, and 20.6 ± 1.7 pA. In the WMN

group, it was 13.9 ± 1.1 pA, 19.6 ± 2.8 pA, and 15.3 ± 1.5 pA. In the STAURO group, it was 16.7 ± 2.0 pA, 19.9 ± 1.7 pA, and 16.2 ± 1.4 pA.

These data suggest that even eight days after the single PAC *in vivo i.p.* treatment, PI3K-signaling plays an important role and may participate in the maintenance of the PAC-induced modulation of TRPV1 receptors. On the other hand, the effect of STAURO on the PAC-reduced tachyphylaxis eight days after the single PAC *i.p.* treatment was not significant. This indicates lesser importance of serine/threonine kinases for the maintenance of PAC-induced changes in this time point.

13.1.6. Wortmannin and Staurosporine *in vivo* Pretreatment Reduced Mechanical Allodynia Present After a Paclitaxel Application

In these behavioral experiments, we tested effects of WMN (0.6 mg/kg; *i.p.* and STAURO (0.1 mg/kg; *i.p.*) pretreatment one hour before the single PAC (8 mg/kg; *i.p.*) injection on PAC-induced mechanical allodynia.

In the first group of animals (Vehicle + PAC; n = 7), the control paw withdrawal threshold (PWT) was 7.4 ± 0.9 g on Day -3 and 7.7 ± 0.3 g on Day 0. The PWT significantly decreased after the PAC treatment ($P < 0.001$; FIG. 13.6.A) on Day 1 (4.4 ± 0.5 g), Day 2 (4.4 ± 0.4 g), Day 3 (3.9 ± 0.4 g), Day 4 (4.1 ± 0.4 g) and Day 8 (4.6 ± 0.4 g). The development of significant mechanical allodynia after the Vehicle + PAC treatment was also confirmed by using individual von Frey filaments. The responsiveness to von Frey filaments with low bending force from 0.16 to 2 grams was increased on each tested day (Day 1, 2, 3, 4 and 8) after the PAC treatment (FIG. 13.6.B).

The second group of animals (pre-WMN + PAC; n = 7) received WMN pretreatment one hour before the single PAC *i.p.* injection on Day 0. The control PWT was 7.4 ± 0.4 g on Day -3 before treatment and 6.8 ± 0.3 g on Day 0; it did not change significantly on Day 1, 2, 3, 4 or 8 in comparison with the control Day 0 FIG. 13.6.C). It was also significantly different from the Vehicle + PAC group on Day 1 (6.5 ± 0.6 g; $P < 0.01$), on Day 2 (6.0 ± 0.5 g; $P < 0.05$), on Day 3 (6.4 ± 0.5 g; $P < 0.001$), and on Day 4 (5.9 ± 0.4 g; $P < 0.05$), but not on Day 8 (5.9 ± 0.4 g; FIG. 13.6.A). The results from both PWT measurements and individual von Frey fibers clearly showed that WMN pretreatment prevented any significant changes in mechanical sensitivity on Days 1–8 after the single PAC treatment (FIG. 13.6.C). This suggests that the inhibition of PI3Ks by WMN pretreatment effectively reduced the development of mechanical allodynia.

The third experimental group (pre-STAURO + PAC; n = 8) was pretreated with STAURO on Day 0. The control PWT on Day -3 and 0 was 7.7 ± 0.3 g and 7.4 ± 0.5 g. The development of mechanical hypersensitivity was significantly prevented only on Day 1 (7.4 ± 0.4 g; $P < 0.001$) when compared with the Vehicle + PAC group (FIG. 13.6.A). However, on Days 2, 3, 4 and 8, the STAURO pretreatment was ineffective; the PWT dramatically decreased compared to control values on Day 1 ($P < 0.001$) and was not different from the Vehicle + PAC treated
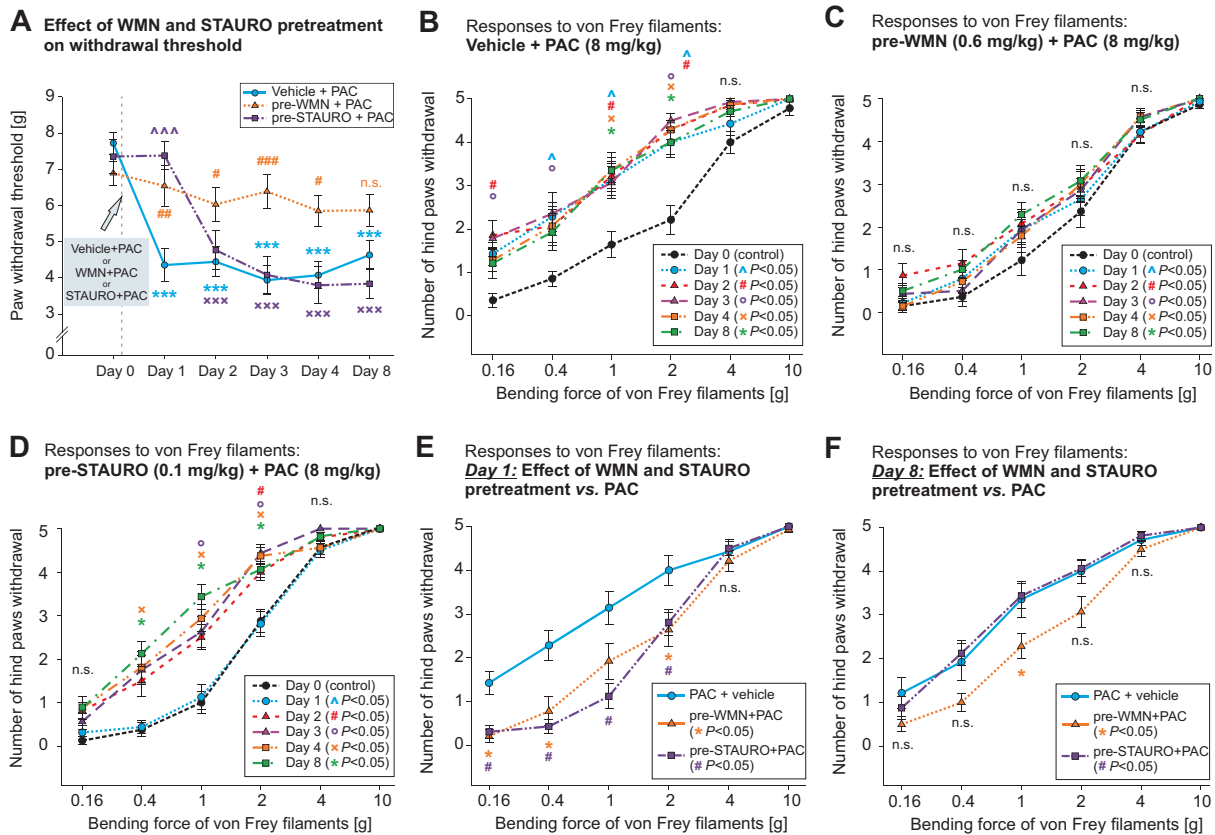
Figure 13.6.: **Changes in mechanical sensitivity induced by the paclitaxel (PAC) treatment and effects of the wortmannin (WMN) and staurosporine (STAURO) pretreatments.** Animals used in this behavioral study were randomly divided into three groups. In total, 22 animals were used. (**A**) The single PAC injection *i.p.* produced significant mechanical allodynia from Day 1 to Day 8 in comparison with the baseline sensitivity (***$P < 0.001$, n = 7). The pretreatment with WMN one hour before the PAC injection significantly reduced allodynia on Day 1 (##$P < 0.01$), Day 2 (#$P < 0.05$), Day 3 (###$P < 0.001$), and Day 4 (#$P < 0.05$; n = 7). The pretreatment with STAURO was effective only on Day 1 ($P < 0.001$), while on Day 2, 3, 4 and 8 the STAURO pretreatment was ineffective and different from the control values on Day 0 (×××$P < 0.001$; n = 8). The Two-Way ANOVA followed by a multiple comparison procedure versus the Vehicle + PAC group and versus Day 0 (the Bonferroni post hoc test) was used for statistical analysis. (**B–F**) Mechanical sensitivity was also measured with six von Frey filaments with bending force from 0.16 to 10 g. (**B**) PAC treatment induced mechanical allodynia from Day 1 to Day 8 (significance for each day and each filament is described in the legend). (**C**) The pretreatment with WMN reduced the development of allodynia from Day 1 to Day 8 and the responses did not differ from the control values (Day 0). (**D**) The pretreatment with STAURO prevented allodynia on Day 1, but it did not change PAC-induced allodynia for the rest of the testing period. The Friedman Repeated Measures ANOVA and Dunn's post hoc test for multiple comparisons versus Day 0 ($P < 0.05$) was used for statistical analysis in **B–D**. (**E**) Both the WMN and STAURO pretreatments significantly inhibited the development of increased sensitivity on Day 1. (**F**) On Day 8 only the WMN pretreatment was effective. The Kruskal-Wallis One-Way ANOVA and Dunn's post hoc test for multiple comparison procedure versus the control group ($P < 0.05$) was used for statistical analysis in (**A–B**).
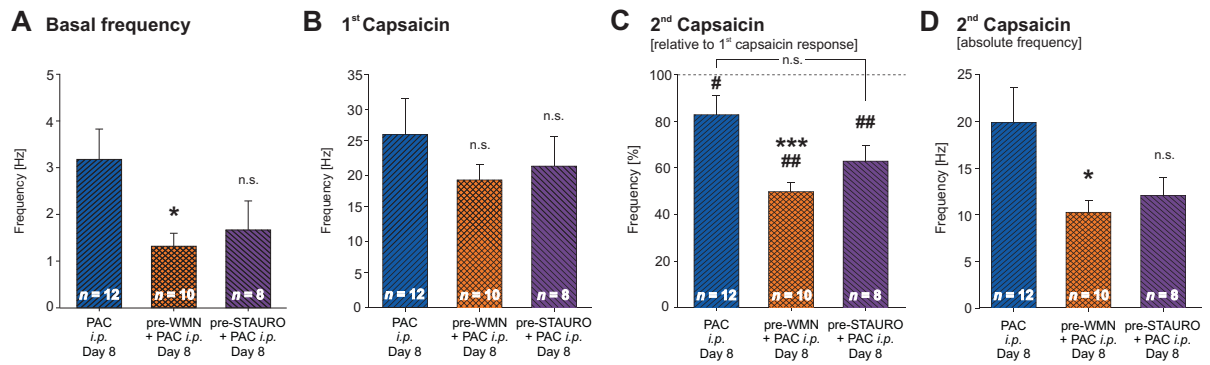
Figure 13.7.: **Effects of the wortmannin (WMN) and staurosporine (STAURO) single *in vivo* treatments before the PAC *i.p.* application on Day 0, on the mEPSCs frequency recorded on Day 8.** (**A**) Neurons recorded on Day 8 after the PAC only treatment had a high basal mEPSCs frequency (3.1 ± 0.6 Hz), while the mEPSCs frequency of neurons in the WMN and STAURO pretreatment groups was much lower (1.3 ± 0.3 Hz (*$P < 0.05$) and 1.7 ± 0.6 Hz, respectively). (**B**) The mEPSCs frequency of the first capsaicin response in the WMN and STAURO groups did not differ from the PAC group of neurons. (**C**) Relative to the first capsaicin response (100 %), the responses to the second capsaicin application were significantly reduced in all groups ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$). However, only the WMN pretreatment was significantly different from the PAC group (***$P < 0.001$), while the effect of the STAURO pretreatment was not. (**D**) The PAC group of neurons had a very high frequency of mEPSCs evoked by the second capsaicin treatment (19.9 ± 3.8 Hz). The response in the WMN group was significantly lower (10.3 ± 1.3 Hz; *$P < 0.05$), while the reduced response in the STAURO group did not reach statistical significance. The One-Way ANOVA followed by the Bonferroni post hoc test, or the nonparametric Wilcoxon signed rank test (**C**) was used for statistical analysis.

group (Fɪɢ. 13.6.A). Similar results were also obtained with the individual von Frey filaments measurements (Fɪɢ. 13.6.D).

Direct comparison of the WMN and STAURO pretreatments on Day 1 and 8 is illustrated in Fɪɢ. 13.6.E and F. Both WMN and STAURO pretreatments significantly inhibited the development of increased sensitivity on Day 1 when compared with the Vehicle + PAC treated group (Fɪɢ. 13.6.E). However, on Day 8, a partial anti-nociceptive effect was present in the pre-WMN + PAC group only (Fɪɢ. 13.6.F).

These data indicate that the inhibition of PI3K with WMN significantly reduced the development of mechanical allodynia for the tested period of eight days after the PAC treatment. The inhibition of serine/threonine protein kinases by STAURO pretreatment reduced allodynia effectively only during the early phase.

13.1.7. Single *in vivo* Pretreatment with Wortmannin, but not Staurosporine, Decreased Paclitaxel-Induced Reduction of the Second Capsaicin Response Tachyphylaxis on Day 8

Some of the animals used in the behavioral experiments were used for the preparation of acute spinal cord slices and for patch-clamp mEPSCs recordings. The aim of these electrophysiological experiments was to verify the difference in the effectiveness of the WMN and STAURO treatments observed on Day 8 in the behavioral study.

First, we compared the effect of the WMN and STAURO in vivo pretreatment on the basal mEPSCs frequency (FIG. 13.7.A). In comparison with the PAC only treated animals (3.1 ± 0.6 Hz; n = 12), both the WMN and STAURO pretreatments reduced the basal frequency of mEPSCs (1.3 ± 0.3 Hz; n = 10, and 1.7 ± 0.6 Hz; n = 8, respecitvely). However, only the WMN pretreatment reduction reached statistical significance compared to the PAC treated group (FIG. 13.7.A; $P < 0.05$).

Responses to the first capsaicin did not differ significantly between the groups (PAC: 26.2 ± 5.5 Hz, WMN: 19.4 ± 2.3 Hz; STAURO: 21.5 ± 4.5 Hz; FIG. 13.7.B).

The second capsaicin response was significantly reduced both in the pre-WMN + PAC (49.8 ± 3.9 %; $P < 0.001$) and in the pre-STAURO + PAC (62.9 ± 6.5 %; $P < 0.001$) groups when compared to the first capsaicin (FIG. 13.7.C). However, when compared to the PAC treated group (82.8 ± 8.3 %), only the WMN pretreatment significantly attenuated the effect of PAC ($P < 0.001$). The analysis of absolute frequencies of the second capsaicin responses revealed similar results (FIG. 13.7.D). In comparison with the PAC only treated group (19.9 ± 3.8 Hz) there was a significant decrease in the pre-WMN + PAC group (10.3 ± 1.3 Hz; $P < 0.05$), but not in the pre-STAURO + PAC group (12.1 ± 1.9 Hz).

The mean amplitude of the mEPSCs did not change significantly under the different experimental conditions (basal activity, first capsaicin, second capsaicin) in any of the examined groups: PAC only treated animals (18.5 ± 1.4 pA, 21.6 ± 1.3 pA, and 20.6 ± 1.7 pA), pre-WMN + PAC (15.5 ± 1.3 pA, 17.3 ± 1.6 pA, and 14.1 ± 0.6 pA), and pre-STAURO + PAC (21.3 ± 3.5 pA, 22.1 ± 1.6 pA, and 17.7 ± 1.3 pA).

Results of these electrophysiological experiments support findings from the behavioral tests obtained in the same animals and validate the strong anti-allodynic effect of the WMN pretreatment.

13.1.8. Paclitaxel *in vivo* Treatment Increased Expression of pAkt in DRG Neurons[3]

Activation of Akt kinase is generally considered as a marker of PI3K signaling pathway activation in the spinal cord or DRG (Pezet *et al.*, 2008; Zhuang *et al.*, 2004). We have analyzed the effect of PAC *in vivo* treatment (8 mg/kg; *i.p.*) on Akt kinase phosphorylation (pAkt; Ser473) in L5 DRG neurons. Representative pictures of immunohistological DRG sections show immunopositivity for pAkt under the Control conditions (FIG. 13.8.A, B), after the PAC treatment (FIG. 13.8.D, E) and after the PAC with WMN pretreatment (FIG. 13.8.G, H). Analysis of the histological data showed that the pAkt positive area (FIG. 13.8.K) and the percentage of pAkt positive cells

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[3] **Author contribution** to presented data adapted from the study Adámek *et al.* (2019). P. Adámek (P.A.) performed the drug administration. Both P.A. and M. Heleš (M.H.) participated in the intracardial perfusion of animals and on tissue dissection for following immunohistochemical processing by the laboratory technician, Mrs. Kateřina Krämerová. M.H. performed image photographing and image analysis. P.A. performed following statistical analysis of the collected dataset, prepared the presented picture, and wrote the draft of the paper.
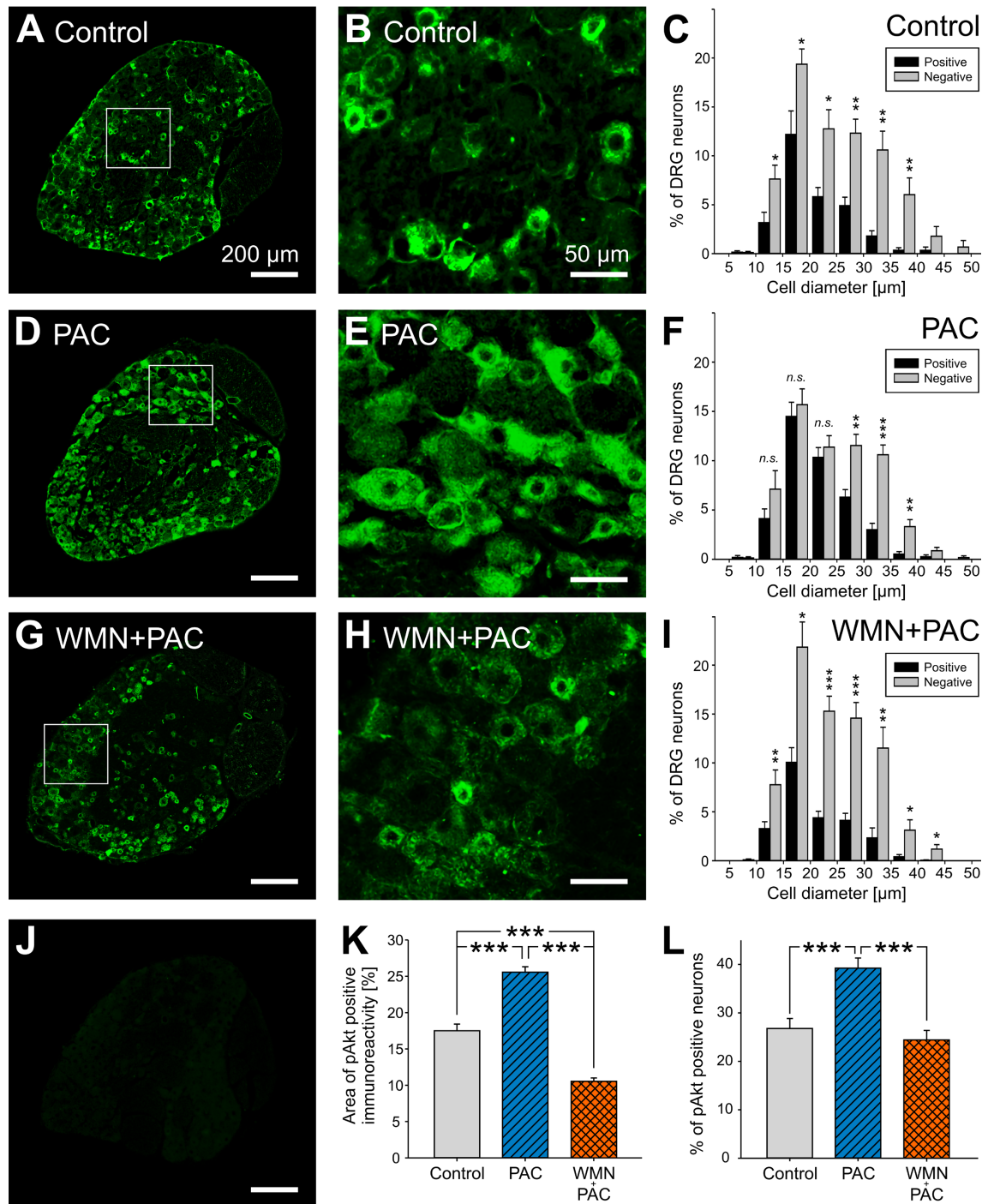
Figure 13.8.: **PI3K inhibitor wortmannin prevented Akt phosphorylation in L5 DRG neurons induced by acute in vivo paclitaxel administration.** A representative picture of phospho-Akt (pAkt) immunofluorescence in the Control group DRG (**A, B**), and size distribution of pAkt positive and negative neurons (**C**) shows pAkt presence in a population of small diameter neurons. (**D, E**) In vivo paclitaxel (PAC) treatment enhanced pAkt immunopositivity and (**F**) the number of positive small diameter neurons (< 25 μm) increased. (**G, H**) Wortmannin pretreatment (WMN + PAC) significantly reduced the effect of PAC on Akt phosphorylation and (**I**) the percentile of pAkt positive neurons was smaller, similar to the Control group. (**J**) The omission of pAkt primary antibody prevented any immunopositivity. (**K**) Area of immunopositivity on the DRG section was significantly higher after the PAC treatment and reduced with the WMN pretreatment. (**A**) The percentage of pAkt positive neurons was increased by PAC and this was prevented by WMN. All data are represented as a mean with SEM. The paired t-test for each size group (**C, F, I**) or the One-Way ANOVA (**K, L**) was used (*P < 0.05; **P < 0.01; ***P < 0.001).

(Fig. 13.8.L) were significantly higher after the PAC treatment. WMN treatment prevented the effect of PAC. More detailed analysis of the DRG neurons (Fig. 13.8.C, F, I) suggested that the PAC application induced increased expression of pAkt especially in the small diameter cells (< 25 µm) as the ratio between the pAkt positive and negative neurons was increased after the PAC treatment in these size categories (Fig. 13.8.F), compared to the Control and WMN + PAC groups (Fig. 13.8.C, I).

13.1.9. Paclitaxel Increases c-Fos expression in the Dorsal Horn Neurons in TRPV1-dependent manner[4]

c-Fos is a small nuclear protein expressed in the dorsal horn neurons as a result of *c-fos* proto-oncogene activation following noxious stimulation and increased activity of peripheral afferent fibers (Coggeshall, 2005). Our immunohistochemical study showed that incubation of rat spinal cord slices in PAC solution (100 nM) for 1 hour significantly increases the number of c-Fos-positive cells in the dorsal horn of the lumbar spinal cord ($P < 0.001$). Pre-incubation of slices with TRPV1 receptor antagonists SB366791 or AMG9810 significantly diminished the PAC-induced c-Fos protein expression ($P < 0.01$). The AMG9810 treatment alone did not show any significant changes in the number of c-Fos expressing dorsal horn neurons from the Control group, while incubation with only SB366791 induced a minor increase in the c-Fos expression ($P < 0.05$; Kalynovska *et al.*, 2017).

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[4] **Author contribution** to presented data adapted from the study Kalynovska *et al.* (2017). N. Kalynovska (N.K.) and J. Paleček (J.P.) designed the experiment; N.K. performed the main part of research; P. Adámek participated on the tissue slices preparation and tissue incubation; the laboratory technician, Mrs. Kateřina Krämerová, made immunohistochemical processing; N.K. analyzed the data; N.K. and J.P. wrote the paper.

13.2. The Study of Disinhibition in Different Pain Models[5]

The balance between inhibitory and excitatory neurotransmission is essential for normal processing and modulation of nociceptive information in the spinal cord dorsal horn. Current literature suggest before that the phenomenon of disinhibition, i.e., loss or deficit in fast GABAergic and glycinergic synaptic transmission in the spinal cord, may be the underlying mechanism of different pain syndromes. Several mechanisms have been proposed for this disinhibition, including the death of inhibitory interneurons, decreased transmitter release, diminished activity of these cells and reduced effectiveness of GABA and glycine as inhibitory transmitters (Todd, 2015; Zeilhofer, 2005; Zeilhofer *et al.*, 2012).

However, despite numerous studies on this important topic, it is still not clear which (if any) of these mechanisms contributes to different pain conditions. Therefore, the aim of our study was to describe and compare changes in inhibitory and excitatory transmission in the superficial laminae (I, II$_o$) excitatory dorsal horn neurons in a model of *paclitaxel-induced peripheral neuropathy* (PIPN), *peripheral inflammation* and in a *chronic constriction injury* (CCI) model of peripheral neuropathy. We used transgenic mice strain *VGAT-ChR2-eYFP*, which expressed *channelrhodopsin-2* (ChR2) under the control of VGAT promoter *Slc32a1* in all inhibitory neurons. Using photostimulation with blue light, this allowed us to selectively, rapidly, and reversibly activate only the population of inhibitory interneurons.

13.2.1. Animal Models of PIPN, Peripheral Inflammation and CCI Reveal Signs of Mechanical Allodynia

PIPN was induced by clinically used PAC given at a dosage of 2 mg/kg intraperitoneally (*i.p.*) every other day for a total 4 injections (days 0, 2, 4 and 6). Final cumulative dose was 8 mg/kg. Peripheral inflammation was induced by subcutaneous injection of 1% carrageenan into both hind paws. CCI was induced by ligation of the sciatic nerve in both hind paws. Mechanical paw withdrawal threshold (PWT) was tested in all experimental animals before the patch-clamp experiment.

The control value of PWT in PIPN group (n = 4; FIG. 13.9.A) was 8.6 ± 0.3 g. PWT decreased significantly even after first PAC i.p. treatment on Day 2 (3.9 ± 0.3 g; P < 0.001) and PIPN was fully developed on Day 7 (2.5 ± 0.3 g; P < 0.001).

In carrageenan group (n = 5; FIG. 13.9.B), the control value of PWT was 7.5 ± 0.4 g and decreased significantly on Day 1 (4.8 ± 0.4 g; P < 0.001).

Animals with CCI (n = 9; FIG. 13.9.C) had control PWT 7.9 ± 0.5 g. PWT decreased significantly on Day 1 (4.0 ± 0.6 g; P < 0.001) and Day 3 (3.6 ± 0.2 g; P < 0.001) after surgery.

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[5] This section contains only preliminary, unpublished data.
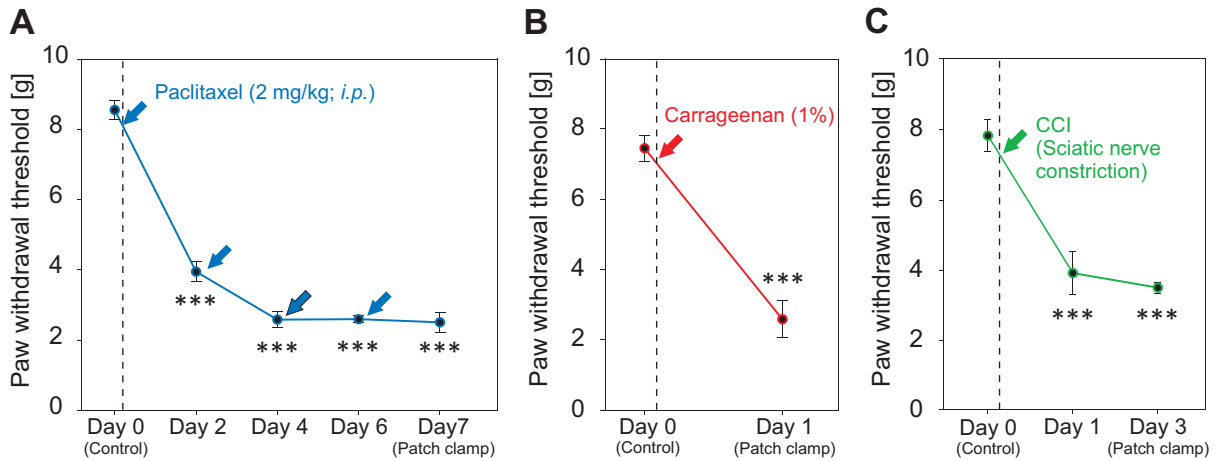
Figure 13.9.: **Mechanical allodynia developed in all experimental models.** Paw withdrawal threshold was significantly decreased in the model of PIPN (**A**), peripheral inflammation (**B**) and after chronic constriction injury of the sciatic nerve (**C**).

13.2.2. Characterization of Excitatory and Inhibitory Neurons

At the beginning of each recording, we tested all neurons for the presence of ChR2 by a 500 ms long light pulse at -70 mV. Fig. 13.10.A shows representative recording of le-IPSC from putative excitatory spinal cord neuron without ChR2-mediated plateau phase of the response[6]

In contrast, Fig. 13.10.B shows typical response of ChR-expressing inhibitory interneuron. This ChR2 mediated response has typical plateau phase and this response is insensitive to VGSCs blocker TTX, $GABA_A$R blocker bicuculline (BIC), GlyR blocker strychnine (STR) or glutamate NMDA and AMPA receptor blocker AP5 and CNQX.

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[6] This light-evoked IPSC is action potential-dependent, and therefore sensitive to TTX. It is mediated by activation of $GABA_A$R and GlyR channels, therefore, it is partly sensitive to BIC or STR and fully inhibitable by co-application of both BIC + STR. Application of glutamate channel blockers CNQX and AP5 did not affect le-IPSC (Data not shown in Fig. 13.10).
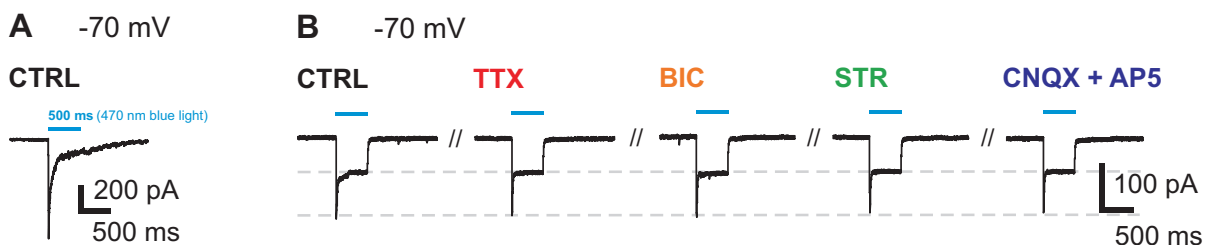
Figure 13.10.: **All neurons were tested for the presence of ChR2 by a 500 ms long light pulse (470 nm) at -70 mV.** (**A**) Typical record from putative excitatory neuron without plateau phase of the response mediated by ChR2. This response was sensitive to bicuculline (BIC) and strychnine (STR). (**B**) The response of ChR2 expressing inhibitory interneuron with the plateau phase of the response present. This response was insensitive to tetrodotoxin (TTX), BIC, STR and also CNQX + AP5 treatment.
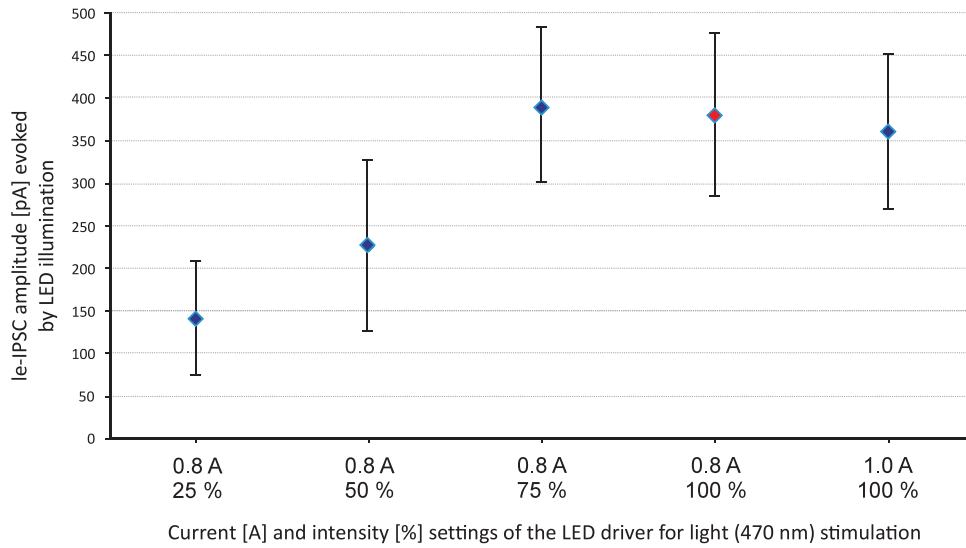
Figure 13.11.: **Experimental determination of supramaximal stimulation for light-evoked IPSC (le-IPSC) stimulation protocol.** We performed voltage clamp recordings of le-IPSC from DH neurons (n = 6) when a series of five different light stimulations with increasing intensities were applied. The following light intensities of the blue light (470 nm) were set on LED trigger and applied on spinal cord slices to evoke le-IPSCs: Current/intensity (I.) 0.8 A/25 %; (II.) 0.8 A/50 %; (III.) 0.8 A/75 %; (IV.) 0.8 A/100 %; (V.) 1.0 A/100 %. We found that LED trigger setting 0.8 A/75 % and higher provides relatively stable le-IPSC responses without further increase. Therefore, we considered the stimulation higher than 0.8 A/75 % as a supramaximal. Hence, the LED trigger setting 0.8 A/100 % (red dot in the graph) was routinely used to evoke stable le-IPSC in all following experiments.

13.2.3. Determination of Supramaximal Stimulation for Recording of Stable Light-Evoked IPSCs

After the characterization of the neuron by 500 ms long blue light stimulation at -70 mV, the recording of sIPSC or le-IPSC was routinely performed at 0 mV and in the presence of glutamate receptors agonists CNQX and AP5.

The aim of this pilot experiment was to establish the appropriate setting of the LED trigger, to induce supramaximal stimulation of inhibitory circuits within the spinal cord slices. We used different settings of input current [A] in combination with different intensities [%].

After establishing of stable whole-cell voltage clamp recording on 0 mV, a series of five different photo-stimulations with increasing intensity of LED light were applied on spinal cord slice. We found that the LED trigger set at 0.8 A/75 % intensity and higher provides stable le-IPSC responses without further increase (FIG. 13.11). We considered the stimulation higher than 0.8 A/75 % as a supramaximal. Hence, the LED trigger setting 0.8 A/100 % (red dot in the graph) was routinely used to evoke stable le-IPSC in all following experiments. We hypothesise that the decrease in amplitudes after stimulation with the two higher intensities (0.8 A/100 % and 1.0 A/100 %, resp.) may be probably a consequence of desensitization or partial depletion of neurotransmitters from synaptic circuits.
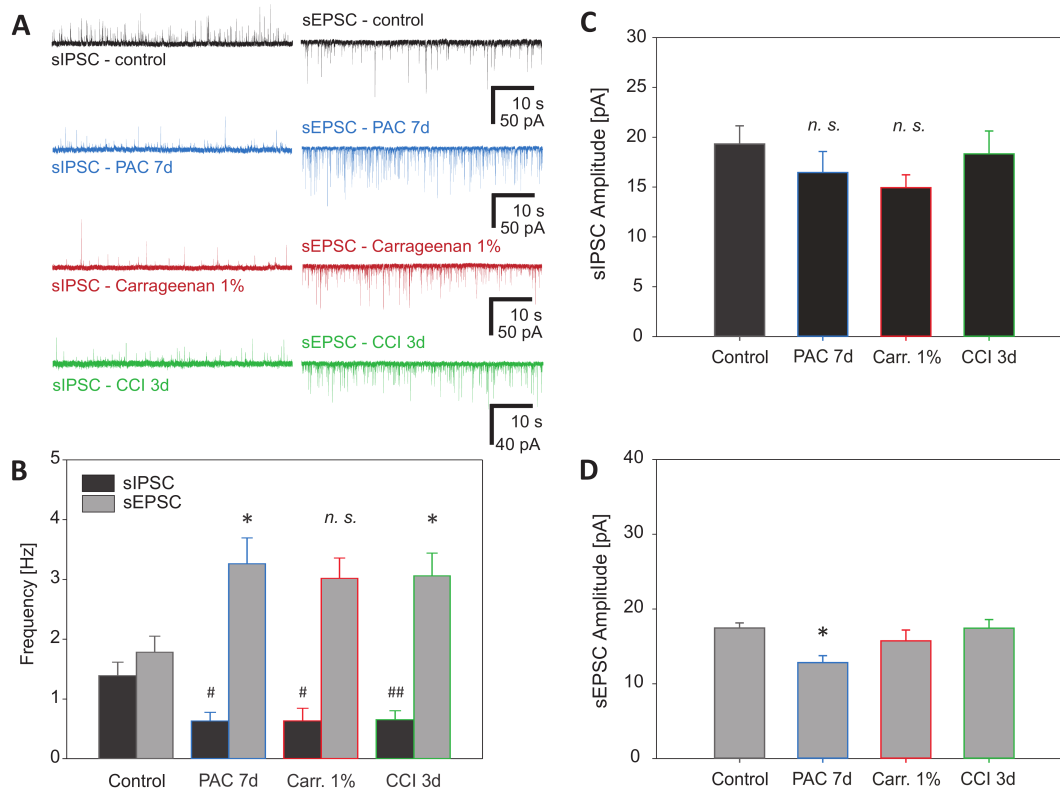
Figure 13.12.: **Frequencies of both sEPSCs and sIPSCs changed significantly in the model of PIPN, peripheral inflammation, and CCI.** Representative traces of sIPSC and sEPSC recorded in superficial excitatory neurons (**A**). The summary graph shows that after paclitaxel treatment, peripheral inflammation and CCI the sIPSC frequency significantly decreased ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$) and sEPSC frequency increased (*$P < 0.05$; **B**). Amplitudes of sIPSC recorded under the pathological conditions did not change significantly (**C**). sEPSC amplitude (expressed as absolute values) was surprisingly lower in neurons from animals after the PAC treatment (**D**). The One-Way ANOVA followed by a multiple comparison procedure versus the control group (the Bonferroni post hoc test) was used.

13.2.4. Frequencies of sIPSCs and sEPSCs Changed in All Pain Models

In the following experiments, we focused exclusively on the changes occurring on excitatory spinal cord DH neurons. Hence, all experimental data described below were obtained only from putative excitatory neurons.

Our preliminary results strongly suggest that sEPSCs are enhanced in all pathological conditions tested (Fig. 13.12.A). The graph in Fig. 13.12.B shows that the sEPSCs frequency increased in all experimental groups compared with the Control group (1.8 ± 0.3 Hz, n = 28), the frequency. It was 3.3 ± 0.4 Hz (n = 18, $P < 0.05$) in the PIPN model on Day 7 following PAC treatment, 3.0 ± 0.3 Hz (n = 16, $P = 0.09$) following 1% carrageen treatment, and 3.1 ± 0.4 Hz (n = 33, $P < 0.01$) following CCI.

In contrast, in all groups were significantly decreased frequencies of sIPSCs compared with control (1.4 ± 0.2 Hz, n = 28). In the PIPN, the frequency was 0.6 ± 0.1 Hz (n = 18, $P < 0.05$), 0.6 ± 0.2 Hz (n = 16, $P < 0.05$) after carrageenan, and 0.7 ± 0.2 Hz (n = 33, $P < 0.01$) in the CCI group.

The amplitudes of sIPSCs did not change significantly in all experimental groups compared to the Control (-17.5 ± 0.7 pA, n = 28, FIG. 13.12.C). The analysis of sEPSCs amplitudes showed small, but significant decrease in PIPN group (-12.8 ± 0.9 pA, n = 18, P < 0.05, FIG. 13.12.D) compared with the Control group. These preliminary results are slightly surprising because it was previously reported that PAC treatment modulates only presynaptic mechanisms (i.e. changed only s/mEPSCs frequency) and did not affect postsynaptic properties (that could be potentially responsible for affecting of postsynaptic currents amplitudes; Adámek *et al.*, 2019; Li *et al.*, 2015a).

13.2.5. The Total Ligh-Evoked Inhibitory Current Decreased in All Pathological States

The aim of the following experiments was to compare the extent to which the total inhibitory input on excitatory neurons is affected by different pathological conditions. We recorded le-IPSCs from excitatory neurons using the supramaximal photostimulation of ChR2 expressing neurons in the spinal cord slices.

The amplitude of total le-IPSC recorded in the Control group of neurons was 745.4 $\pm$ 82.2 pA (n = 27, FIG. 13.13.A and B). Under all pathological conditions le-IPSC significantly decreased; in PIPN group the amplitude of le-IPSC was 486.4 ± 51.8 pA (n = 18, P < 0.05); in carrageenan group the amplitude of le-IPSC was 446.0 ± 74.6 pA (n = 16, P < 0.01); and finally 369.4 ± 35.6 pA (n = 33, P < 0.001) in CCI group (FIG. 13.13.A and B). Excluding the carrageenan group, the area under the curve of le-IPSC was also significantly changed (P < 0.001; FIG. 13.13.C). This may suggest that the properties of the postsynaptic receptor may be changed. However, the detailed analysis of decay and rise time did not reveal any changes (FIG. 13.13.E and F). To a more detailed description of le-IPSC, the time constant *Tau* was fitted. le-IPSCs were best fitted with standard bi-exponential function with the Chebyshev method using Clampfit 10.5. software. Time constants *Tau* were determined for both slow (putative GABAergic) and fast (putative Glycinergic) component of the le-IPSC. Time constants did not change significantly in any group (FIG. 13.13.G). Absolute amplitudes of both components substantially decreased compared to the control group (FIG. 13.13.H), which correlates with the finding that amplitude of the total Ie-IPSC (FIG. 13.13.B) decreased. However, when absolute amplitudes of each component were standardized as % of the total current, no changes between the groups were detected (FIG. 13.13.I).

These data suggest that in all pathological conditions tested the disinhibition occurred in a significant manner. However, these data do not clearly answer the question whether presynaptic or postsynaptic mechanisms are mainly involved. Therefore, it is necessary to perform more detailed experiments, e.g., paired-pulse ratio stimulation to define the role of pre- or postsynaptic mechanisms in our experiments.
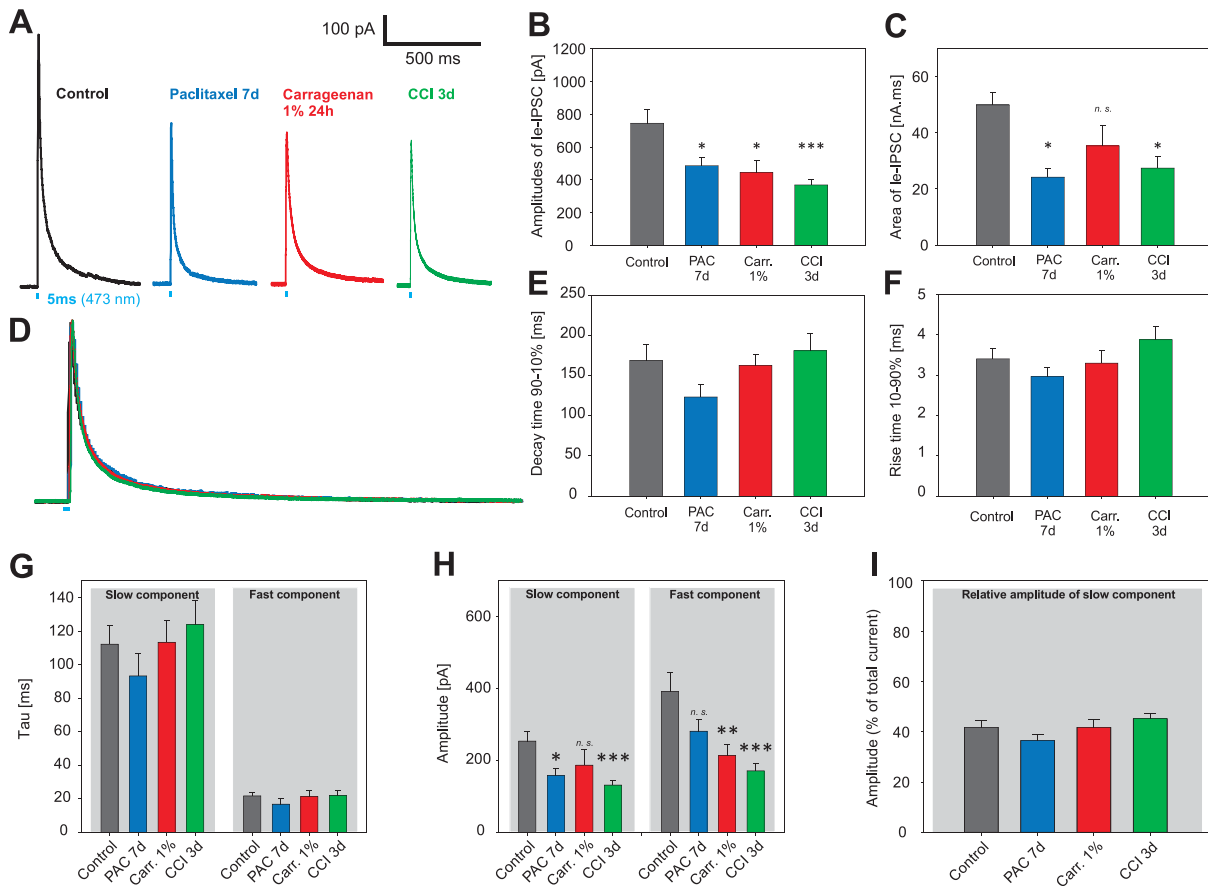
Figure 13.13.: **Amplitudes of light-evoked IPSC (le-IPSC) decreased under the pathological conditions.** (**A**) Representative light-evoked IPSC (le-IPSC) from the control group and from different experimental models. (**B**) The amplitude of the le-IPSC had significantly decreased in neurons from paclitaxel, carrageenan and CCI models. (**C**) Paclitaxel and CCI decreased significantly also the area of le-IPSC. (**D, E, F**) Decay and rise time of le-IPSC did not change significantly in any group. (**G**) Time constant *Tau* was determined for both slow (putative GABAergic) and fast (putative Glycinergic) component of the le-IPSC. le-IPSCs were best fitted with standard bi-exponential function with the Chebyshev method using Clampfit 10.5. software. Time constant did not change significantly in any group. (**H**) Absolute amplitudes of both components decreased substantially. (**I**) When the amplitudes of both slow and fast component were standardized as % of the total current, there were no changes present. Only slow component is ploted, fast is complementary to 100 %. The One-Way ANOVA followed by a multiple comparison procedure versus the control group (the Bonferroni post hoc test) was used.

13.3. Inhibition of the Na$_v$1.7 by Protoxin II Reduces Burn Injury-Induced Spinal Nociceptive Signaling

Recent findings suggest that reducing the activity of VGSC Na$_v$1.7 in primary sensory neurons could improve pain in different pathological states. Therefore, we tested in wide collaboration with the team of Prof. ISTVAN NAGY (Imperial College London) the effect of Na$_v$1.7 antagonist Protoxin II (ProTxII) on nociceptive synaptic processing after burn injury.

13.3.1. ProTxII Application Reduces the sEPSCs Frequency After Burn Injury[7]

To confirm that tarantula venom-peptide ProTxII reduces spinal nociceptive processing in burn injury model, we assessed the effect of ProTxII on sEPSCs in the superficial spinal cord dorsal horn neurons.

The sEPSCs frequency in the sham-operated animals was 0.8 ± 0.2 Hz (n = 9) and it did not change after the ProTxII (10 nM) acute application compared to the control frequency (99.7 ± 8.7 % of the control value; FIG. 13.14.Aa, B). sEPSCs amplitudes in the sham-operated group were -14.7 ± 2.0 pA before and -15.1 ± 2.2 pA after the ProTxII application and it was not significantly different. All the tested neurons responded to capsaicin (200 nM) application with a robust and significant increase of sEPSCs frequency (25.6 ± 5.7 Hz, n = 8; P < 0.01). It confirms their involvement in spinal nociceptive circuits.

The sEPSCs frequency showed a robust and significant increase following burn injury (3.1 ± 0.6 Hz; FIG. 13.14.Ab, B; P < 0.01). Application of ProTxII induced a significant decrease of the sEPSC frequency (2.1 ± 0.5 Hz; Fig. 13.15 Ab, B), which represent 66.2 ± 8.1 % of the control value. The averaged amplitude of the sEPSCs was -17.8 ± 3.4 pA and did not change after ProTxII treatment (-17.1 ± 3.6 pA). All of the neurons showed a significant increase of the sEPSCs frequency after capsaicin application (31.5 ± 7.1 Hz; n = 10; p < 0.01). The capsaicin response was not different in the sham-operated and burn-injured groups.

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[7] **Author contribution** to presented data adapted from the study Torres-Peréz *et al.* (2018). P. Adámek (P.A.) conducted all of the electrophysiological recordings and the subsequent data analysis of sEPSCs presented in this section. P.A. participated in the writing of the paper.
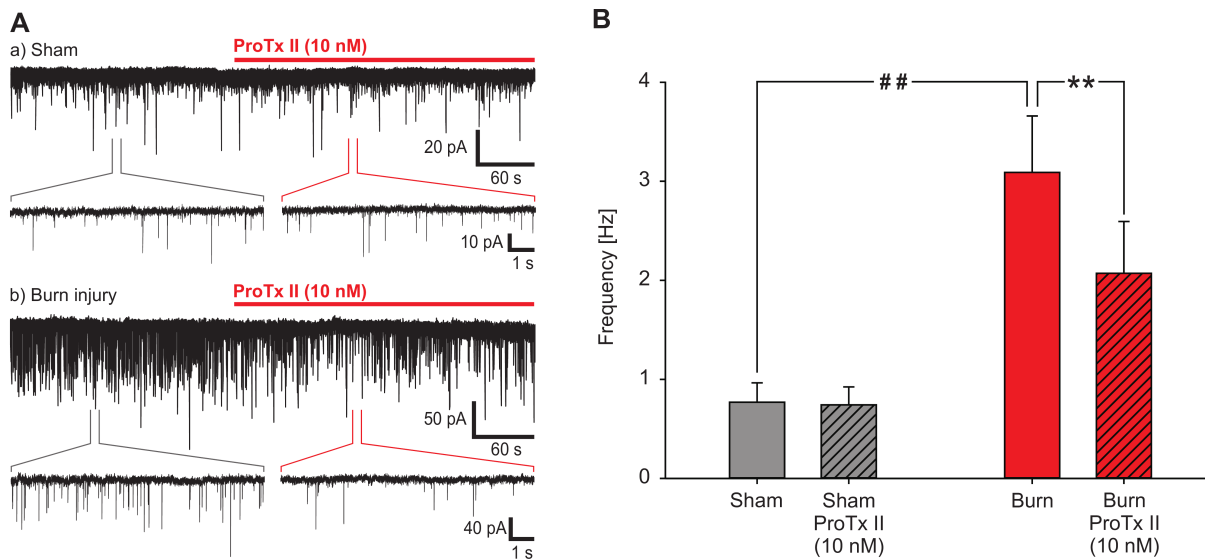
Figure 13.14.: **Effect of the ProTxII application on spontaneous EPSC frequency recorded from spinal superficial dorsal horn neurons.** In the slices with the sham treatment (n = 9), the basal sEPSCs frequency was low and ProTxII (10 nM) treatment did not evoke any change, as is evident on the representative native recording (**Aa**, sham) and from the averaged responses (**B**). However, neurons in slices prepared after the burn injury (n = 10) had robust spontaneous activity (**Ab**, burn injury) and application of ProTxII induced a significant decrease of the sEPSC frequency (**A**, **B**, **P = 0.006). The basal sEPSCs frequency between the sham group and the burn injury group was significantly different (B, ##P = 0.002). Statistical significance was tested using paired t-test within each group (**P < 0.01), and by t-test with Bonferroni correction for two comparisons between sham and burn injury group (##P < 0.005).

13.4. Modulation of Nociceptive Synaptic Transmission by Anandamide Precursor 20:4-NAPE[8]

The last part of the results is devoted to the modulatory role of endogenous lipid precursor of anandamide (AEA), *N*-arachidonoylphosphatidylethanolamine (20:4-NAPE). The presented results represent a part of the study of Nerandžič *et al.* (2018), which reported the altered mechanism of 20:4-NAPE modulatory effect of on spinal cord transmission after peripheral inflammation.

13.4.1. Incubation of Spinal Cord Slices in 20:4-NAPE Containing Solution Leads to Increased Anandamide Production

The first aim of our study was to verify the production of AEA from 20:4-NAPE in our preparation. Mass spectrometry analysis was used to quantification of AEA content in our samples after incubation of spinal cord slices *in vitro* in different concentrations of 20:4-NAPE (10, 100, 200 µM). Under the control conditions with the extracellular solution only, the average AEA concentration in the solution was very low (7067 $\pm$ 4532 of peak area). AEA concentration increased gradually with increasing concentration of precursor 20:4-NAPE (20 µM: 48.324 $\pm$ 27.502; 100 µM: 103.310 $\pm$ 38.179; 200 µM: 298.004 $\pm$ 139.867 AEA peak area). To reduce the differences between the individual experiments and to facilitate statistical analysis, these results were normalized against the content of AEA found after the incubation with the highest concentration of 20:4-NAPE (200 µM; set as 100%; Fig. 13.15). There was no AEA detected in the samples where 20:4-NAPE was present without the slices. These results indicate that 20:4- NAPE (20 µM) application to the spinal cord slices led to increased anandamide concentration in the slice.

13.4.2. The Role of CB$_1$ Receptors in 20:4-NAPE-mediated Inhibition in the Model of Peripheral Inflammation

This experiment builds on previous findings from our laboratory that 20:4-NAPE reduce the frequency of sEPSCs. In spinal cord slices prepared from naive animals was this effect dependent on the activation of CB$_1$ receptors, but not TRPV1 (Nerandžič *et al.*, 2018; *Fig. 3*).

Therefore, the aim of the following experiment was to answer the question, which role plays these receptors in the model of peripheral inflammation.

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[8] **Author contribution** to presented data adapted from the study Nerandžič *et al.* (2018). P. Adámek (P.A.) dissected and incubated spinal cord tissue for anandamide production for subsequent mass spectrometry analysis, performed by Prof. Ivan Mikšík. P.A. participated in the acute inflammatory model preparation and data acquisition of the electrophysiological recordings of sEPSCs presented in this section.
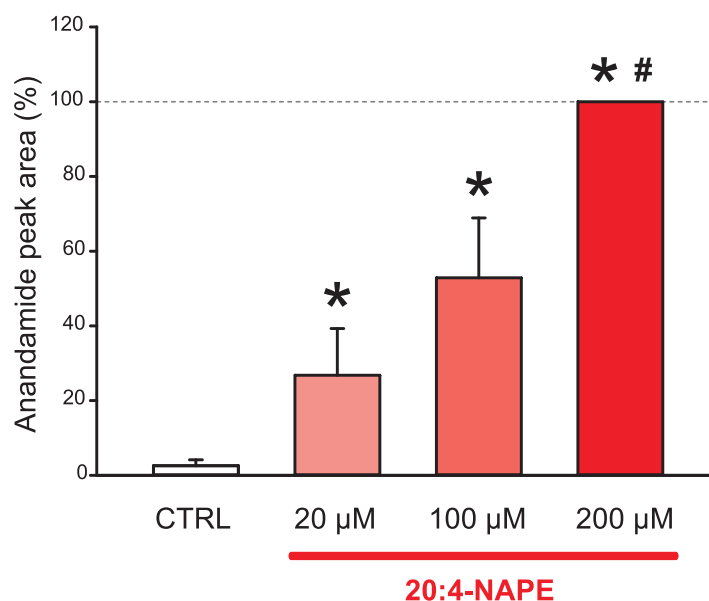
Figure 13.15.: **AEA concentration increases after incubation with 20:4-NAPE.** Three different concentration of 20:4-NAPE (20, 100, 200 µM) were used for incubation with spinal cord slices. An increasing amount of AEA was detected in extracellular solution after NAPE application, in a concentration-dependent manner. Statistical significance was tested using repeated measures ANOVA on ranks followed by Student–Newman–Keuls test; n = 5); *$P < 0.05$, significantly different from control, #$P < 0.05$, significantly different from 20 and 100 µM 20:4-NAPE.

The role of CB_1 was studied using antagonist PF514273 (0.2 µM). The pretreatment with PF514273 (6 min) alone did not change the basal sEPSCs frequency (108.0 ± 9.8 %; n = 16; FIG. 13.16.A, B). In addition, the amplitudes were not significantly affected by PF514273 application alone (24.6 ± 2.5 pA) compared with control sEPSCs (27.4 ± 2.4 pA).

Similarly to control conditions, co-application of CB_1 inhibitor PF514273 with 20:4-NAPE substantially abolish the 20:4-NAPE-mediated inhibition in the slices prepared from inflammatory animals. Moreover, there is substantial increase in sEPSCs frequency (148.8 ± 16.8 %, n = 16; FIG. 13.16.A, B). This increase was not significantly different in comparison with control frequency; however, it is significantly different ($P < 0.05$) from the effect of 20:4-NAPE alone (Nerandžič *et al.*, 2018; *Fig. 6A*; $P < 0.05$). The amplitude of sEPSCs was not changed after co-application of PF514273 with 20:4-NAPE coapplication (24.1 ± 2.2 pA; n = 16).

In the context of the entire study Nerandžič *et al.* (2018), these data suggest that the inhibitory effect of 20:4-NAPE on sEPSCc frequency is under inflammatory condition mediated by CB_1 receptors activation, similarly to control. The potentiating effect of 20:4-NAPE on sEPSCs visible in representative recording in FIG. 13.16.A, and in graph FIG. 13.16.B was prevented by blockade of TRPV1, which are following inflammation tonically activated. This effect is unmasked just following CB_1 inhibition (Nerandžič *et al.* 2018). This issue will be discussed in more detail in the discussion.
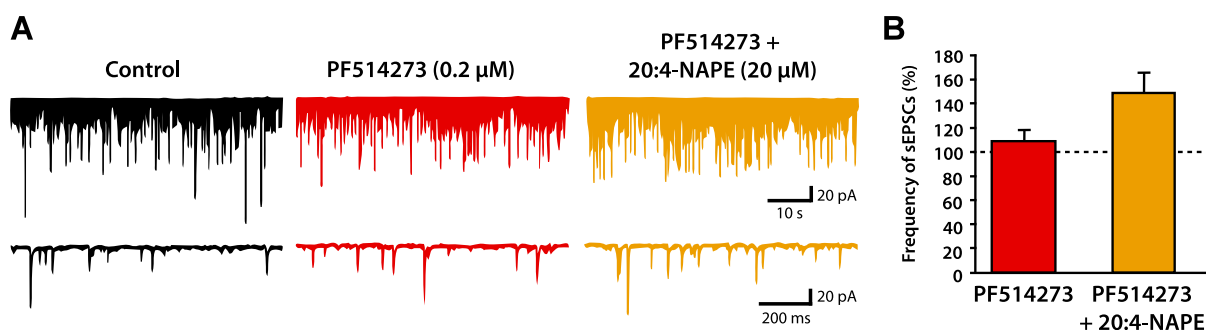
Figure 13.16.: **The effect of CB$_1$ antagonist PF514273 on 20:4-NAPE-induced inhibition of sEPSCs frequency under inflammatory conditions.** Representative sEPSCs recording (**A**) shows that the application of PF514273 (0.2 µM) alone was not changed the sEPSCs frequency. Co-application of PF514273 with 20:4-NAPE 20 (µM) abolished the 20:4-NAPE-mediated inhibition (**A**, **B**). Moreover, inhibition of CB$_1$ unmasked the potentiating effect of 20:4-NAPE mediated by TRPV1 channels, which is significantly increased compared to the effect of 20:4-NAPE alone (see a discussion or Nerandžič *et al.* 2018 in an Appendix). Statistical significance was tested using One-Way repeated measures ANOVA followed by Student-Newman-Keuls test.

14. Discussion

14.1. The role of TLR4, TRPV1, and PI3K in Paclitaxel-Induced Neuropathic Pain

The anti-neoplastic effect of PAC used in chemotherapy is based primarily on the ability of PAC to bind to the cytoskeletal protein tubulin (Arbuck *et al.*, 1993). However, the modulation of apoptotic processes by activation of the MAPKs pathways has also been documented (MacKeigan *et al.*, 2000; McDaid & Horwitz, 2001), and taxanes, including PAC, also display immunomodulatory effects (Fitzpatrick & Wheeler, 2003). Agonistic effect of PAC on TLR4 activation in rodents has been reported repeatedly and it is currently accepted by the scientific community (Ding *et al.*, 1990; Fitzpatrick & Wheeler, 2003; Javeed *et al.*, 2009; Li *et al.*, 2014; Yan *et al.*, 2015b).

Our results demonstrated that PAC can similarly to potent TLR4 agonist LPS (Diogenes *et al.*, 2011) directly activate peripheral sensory and spinal neurons and sensitize them to capsaicin-induced TRPV1-mediated responses. We showed in collaboration with our colleagues from The University of Texas M.D. Anderson Cancer Center that a direct functional interaction between TLR4 and TRPV1 occurred in rat and human DRG, TLR4/TRPV1-transfected HEK293 cells and both in rat and mouse spinal cord slices (Li *et al.*, 2015a). Both acute *in vitro* and systemic *in vivo* PAC treatment increases mEPSCs, respectively sEPSCs frequency, which indicates an increase in the release of glutamate from presynaptic endings in the dorsal horn of the spinal cord (Li *et al.*, 2015a). The TLR4-activation-dependent effect of acute PAC treatment has been reported also by Yan *et al.* (2015). Excessive activation of excitatory glutamatergic synaptic transmission in the dorsal horn is considered as one of the most important mechanisms, involved in abnormal neuronal activation in different pathological pain conditions (Chen *et al.*, 2009; Kuner, 2010; Špicarová *et al.*, 2014a; Špicarová *et al.*, 2011).

In our following experiments with PAC, we showed that treatment of spinal cord slices with PAC *in vitro* enhances expression of the c-Fos protein in TRPV1-inhibitable manner (Kalynovska *et al.*, 2017), suggesting the induction of sensitization of the dorsal horn neurons (Chen *et al.*, 2009; Kuner, 2010; Špicarová *et al.*, 2014a; Špicarová *et al.*, 2011).

Moreover, we have recently demonstrated for the first time that PI3K signaling plays an important role in the early development and maintenance of mechanical allodynia in an animal model of PIPN. We showed that single administration of wortmannin pretreatment *in vivo* significantly attenuated increased mechanical sensitivity for up to eight days after the PAC treatment, and it also prevented enhancement of TRPV1-mediated responses to repeated
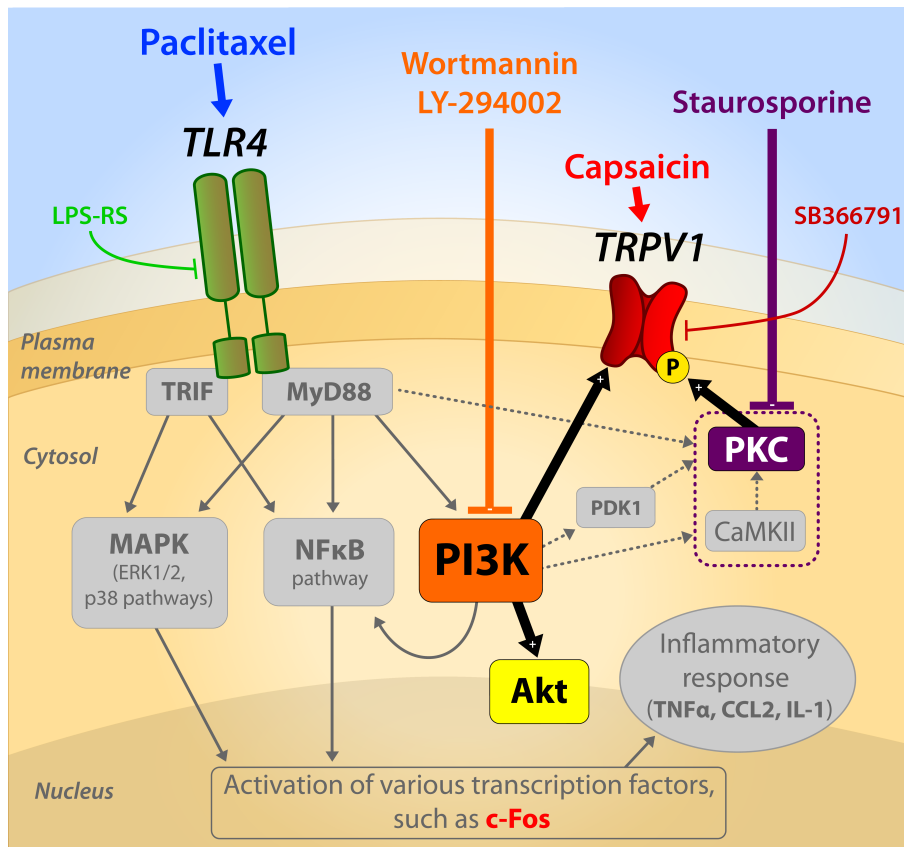
Figure 14.1.: **Schematic diagram summarizing the proposed mechanism of the paclitaxel-induced modulation of the TRPV1 receptors function in nociceptive DRG neurons.** For clarity, signaling tested in our studies is in color, while other signaling pathways are in gray. Signal transduction following the TLR4 activation occurs via two adapter signaling molecules MyD88 (Myeloid-differentiation response gen 88) and TRIF (Toll/interleukin-1 receptor (TIR) domain-containing adapter-inducing interferon-β). The PI3K signaling pathway may be stimulated via MyD88-dependent mechanism after the TLR4 activation (Li *et al.*, 2003). The TLR4 activation may also activate PKC (the dotted lines) via MyD88 (Faisal *et al.*, 2008), or PKC may be activated by PI3K via CaMKII (Bonnington & McNaughton, 2003) or via PDK1 (Dutil *et al.*, 1998). Our experiments showed that PI3K inhibitors wortmannin, LY-294002 and serine/threonine kinases inhibitor staurosporine prevented the paclitaxel-induced changes in tachyphylaxis of capsaicin-induced responses (Adámek *et al.*, 2019).

applications of capsaicin in the electrophysiological recordings. Results from *in vitro* experiments with acute PAC applications, together with PI3K antagonists supported the *in vivo* results in all of the tested situations. Blocking the activity of serine/threonine kinases (such as PKC, PKA, and CaMKII) by the staurosporine treatment was effective in the acute *in vitro* experiments and on the first day of the behavioral study, but it did not have a significant impact later—eight days after the PAC application (Adámek *et al.*, 2019).

In contrast to observations in rodents, the effect of PAC on human TLR4 is controversial, as there is circumstantial evidence that the TLR4 accessory protein MD-2 is necessary for PAC-mediated TLR4 activation in mice, but not in human cells (Kawasaki *et al.*, 2001; Resman *et al.*, 2008; Zimmer *et al.*, 2008). However, PAC was shown to activate TLR4 signaling, leading to enhanced TRPV1-mediated capsaicin responses in human DRG neurons and in HEK293 cells co-expressing human TRPV1 and TLR4 receptors (Li *et al.*, 2015a).

Signal transduction following TLR4 activation occurs via two immediate adapter molecules MyD88 and TRIF (FIGURE 14.1, p. 118). These two adapter proteins are linked to the activation of NF-κB (Takeda & Akira, 2004) and MAPKs including ERK1/2, JNK, and p38 kinase (Guha & Mackman, 2001). Both MyD88 and TRIF signaling pathways play an important role in PAC-induced peripheral neuropathy (Byrd-Leifer *et al.*, 2001; Li *et al.*, 2014).

It has been reported recently, that NFκB and MAPK signaling downstream to TLR4 contributes to PAC-related CIPN. Expression of ERK1/2 and P38, but not JNK MAPK has increased in DRG nociceptive neurons (Li *et al.*, 2015b). Recently, it has been also shown that TLR/MyD88 signaling play an important role in neuroinflammation and persistent neuropathic pain after chronic constriction injury (Liu *et al.*, 2016).

Moreover, the PI3K/Akt signaling pathway is involved downstream to the TLR4 (Guha & Mackman, 2001; Ojaniemi *et al.*, 2003). All four class I PI3K isoforms (α, β, γ, δ) are differentially expressed in the spinal cord, DRG, and peripheral nerves in naive animals (Leinders *et al.*, 2014). The PI3K pathway (FIGURE 14.1, p. 118) may be stimulated via a MyD88-dependent mechanism after TLR4 activation (Li *et al.*, 2003). The activation of PI3K via TLR4 mechanisms could facilitate trafficking of TRPV1 receptors into the plasmatic membrane, similar to the NGF- and BDNF-induced TrkA (Tropomyosin receptor kinase A)/PI3K signaling pathways (Duan *et al.*, 2012; Stein *et al.*, 2006).

Direct interaction between TRPV1 and PI3K contributes to enhanced trafficking of TRPV1 to the plasma membrane (Stein *et al.*, 2006; Zhu & Oxford, 2007). Also, the PI3K-mediated conversion of phosphatidylinositol (4,5)-bisphosphate (PIP$_2$) to phosphatidylinositol (3,4,5)-bisphosphate (PIP$_3$) may contribute to the sensitization of TRPV1 (Cao *et al.*, 2013).

Although no changes were detected in the expression of the PI3K/Atk pathway after the PAC treatment (Li *et al.*, 2015b), it does not exclude the possibility that TLR4/PI3K signaling modulates TRPV1 receptor sensitivity to capsaicin after the PAC treatment. The PI3K-mediated increase of TRPV1 trafficking into the plasmatic membrane could explain reduced tachyphylaxis of the second capsaicin response in our experiments with the acute PAC treatment. It would also correspond to the increased mEPSCs frequencies induced by the first capsaicin application after the *in vivo* PAC treatment. The role of PI3K in this process was clearly demonstrated by the strong effect of both PI3K inhibitors in our acute experiments. We would expect that the described PAC-induced effects on the TRPV1-mediated capsaicin responses in our experiments were mediated predominantly by presynaptic mechanisms, as we did not observe any significant changes in the mEPSCs amplitudes, as was reported previously (Yan *et al.*, 2015b).

The PAC-induced increase of mechanical (Braz *et al.*, 2015; Hara *et al.*, 2013; Li *et al.*, 2015a; Yadav *et al.*, 2015; Yan *et al.*, 2015b; Zhang *et al.*, 2013) and thermal (Braz *et al.*, 2015; Cata *et al.*, 2006; Hara *et al.*, 2013; Yadav *et al.*, 2015) sensitivity is well documented and our behavioral results after the *in vivo* PAC application are in good correlation with that, taking into account the different dosing and PAC concentrations. We reported that the single

systemic wortmannin pretreatment one hour before the PAC administration significantly dimin- ished the development of mechanical hypersensitivity on all the tested days (1, 2, 3, 4 and 8) after the treatment; it also prevented the changes in tachyphylaxis of the second capsaicin re- sponse in spinal cord slices prepared from these animals on Day 8. The same single systemic staurosporine pretreatment was effective only on Day 1 in preventing mechanical allodynia after the PAC *in vivo* application; it did not have an effect on Days 2, 3, 4 and 8, when mechanical sensitivity was similar to the PAC treated groups. In the electrophysiological experiments on slices from these animals, we did not see any significant changes in capsaicin responses on Day 8, compared to the PAC treated controls. However, the application of staurosporine in acute elec- trophysiological experiments clearly demonstrated a strong effect in preventing the PAC-induced reduction of the second capsaicin response tachyphylaxis.

It was shown previously that TLR4 activation may activate PKC (FIGURE 14.1, p. 118) via the scaffold protein MyD88 (Faisal *et al.*, 2008). PKC may also be activated by PI3K via CaMKII (Bonnington & McNaughton, 2003) or via PDK1-dependent mechanism (Dutil *et al.*, 1998). Therefore, we suggest that serine/threonine kinases such as PKC, PKA, and CaMKII that are inhibited by staurosporine are involved in the early development of the PAC-induced CIPN and that they also play a role in tachyphylaxis of the capsaicin response present after the acute PAC application. This is in agreement with the well-documented role of these kinases in the modulation of TRPV1 function (Bhave *et al.*, 2003; Bhave *et al.*, 2002; Bonnington & McNaughton, 2003; Nagy *et al.*, 2014; Zhu & Oxford, 2007).

In our behavioral study, we used a single pretreatment with wortmannin and staurosporine. It is possible that different pharmacokinetic properties of these drugs could play a role in their different efficiency in preventing allodynia. Wortmannin has been reported to be very unstable in biological fluids with a half-life between 8–13 min (Holleran *et al.*, 2003; Stein & Waterfield, 2000). Despite this, many articles describe a long-term effect (hours to days) of the wortmannin treatment under *in vivo* conditions (Liu *et al.*, 2018; Sun *et al.*, 2006; Xu *et al.*, 2014; Xu *et al.*, 2007; Yuan *et al.*, 2007). This prolonged biological activity of wortmannin is caused by its unusual, reversible covalent reaction with nucleophiles (e.g. negatively charged amino acids) in biological fluids (Yuan *et al.*, 2007). This so-called *"wortmannin paradox"* may provide an ex- planation for the long-term effect of the single *in vivo* wortmannin treatment in our experiments. The half-life of staurosporine in rats is ∼51 min in plasma and staurosporine is highly adsorbed by endothelium after *in vivo* administration (Gurley *et al.*, 1998). The relatively short-lasting effect of the staurosporine treatment after the single *in vivo* administration could be caused by its low concentration at the time when PAC could still be present.

These results suggest that the reported changes in tachyphylaxis of the second capsaicin response in acute conditions after the PAC application may be generated by both PI3K- and PKC-mediated mechanisms such as TRPV1 receptors trafficking into the plasma membrane and their phosphorylation. However, under more chronic conditions (8 days after the PAC treatment), these changes are more likely to be maintained by a PI3K-dependent mechanism.

Reported changes in TRPV1 receptors tachyphylaxis on Day 8 after the PAC treatment may also be modulated by other mechanisms. Low concentrations of PAC penetrate into the cerebrospinal fluid and the spinal cord after one PAC application and are detectable 24 hours later (Yan *et al.*, 2015b). It is possible that in the later stages, canonical TLR4 signaling pathways activated by the initial PAC activation play a role. The activation of NFκB downstream to TLR4 in macrophages and endothelial cells (Jones *et al.*, 2007; Li *et al.*, 2003) can lead to the release of pro-inflammatory cytokines that were reported as modulators of TRPV1 receptors (Gao *et al.*, 2009; Špicarová *et al.*, 2014a; Špicarová & Paleček, 2010). Also the activation of TLR4 in primary sensory neurons in the lumbar DRG may lead to the regulation of expression of pro-inflammatory cytokines and to increased levels of CCL2 and CCR2 receptors (Zhang *et al.*, 2013), TNFα (Byrd-Leifer *et al.*, 2001; Ledeboer *et al.*, 2007), and IL-1β (Ledeboer *et al.*, 2007) after the PAC treatment. Another potential advantage of PI3K signaling inhibition could be the prevention of blood-brain barrier disruption with PI3K inhibitors (Camire *et al.*, 2014), which could decrease the availability of PAC in the central nervous system.

Another clinically important consequence of our findings is related to PAC-induced chemoresistance. Chemoresistance to PAC is reported as another common clinical problem in chemotherapy. Earlier reports showed that activation of JNK and p38 MAPK are critical for induction of apoptosis, whereas activation of ERK1/2 has an anti-apoptotic effect and leads to proliferation and cell survival (Xia *et al.*, 1995). All of these mentioned MAPK signaling pathways are also involved in TLR4 signaling (Guha & Mackman, 2001). Inhibition of ERK1/2 pathway components in combination with PAC was shown effective in enhancement of tumor cell apoptosis (Kawaguchi *et al.*, 2007; MacKeigan *et al.*, 2000, 2002; McDaid & Horwitz, 2001). Besides ERK1/2, anti-apoptotic effect is also attributed to PI3K/Akt/NFκB pathway (Mabuchi *et al.*, 2004), because several studies have shown that inhibition of PI3K signaling with inhibitors wortmannin and LY-294002 synergistically increased the efficacy of PAC-induced apoptosis in many cancer cell lines (Kawaguchi *et al.*, 2007; Kim *et al.*, 2007; MacKeigan *et al.*, 2002). WMN exert also marked inhibition of pancreatic cancer cell migration and metastasis *in vivo* (Teranishi *et al.*, 2009). It was reported, that inhibition of PI3K/Akt signaling with WMN increases also the efficacy of cisplatin treatment in *in vivo* ovarian cancer model (Ohta *et al.*, 2006). Taken together with our present findings, the usage of PI3K inhibitors may potentially improve both PAC-induced neuropathic pain states and may increase the therapeutic efficacy of PAC-chemotherapy. Therefore, we suggest that inhibition of PI3K signaling may be a potentially advantageous new strategy for cancer chemotherapy per se because inhibition of PI3K attenuate neuropathic pain states and it also may attenuate chemoresistance of cancer cells.

In conclusion, our results showed that the presence of mechanical allodynia after the PAC treatment could be prevented by the inhibition of PI3K pathways. This behavioral effect of PI3K antagonist was also accompanied by reduced responsiveness of spinal TRPV1 receptors. We suggest that the inhibition of PI3K signaling may be a potentially advantageous promising new strategy to attenuate neuropathic pain states after chemotherapy treatments.

14.2. Disinhibition as a Significant Player in the Pathology of Different Pain States

A fine balance between excitatory and inhibitory neurotransmission of primary afferent fibers and inhibitory interneurons in the spinal cord dorsal horn is considered essential for processing and modulation of nociceptive information (Zeilhofer *et al.*, 2012). It was suggested that lose or attenuation of the inhibitory component, i.e., disinhibition of fast GABAergic and glycinergic synaptic transmission, contribute to the development of neuropathic and inflammatory pain states much more than the increased activity of primary sensory afferents (Zeilhofer, 2005). However, the exact mechanisms of inhibitory synaptic processing modulation under pathological conditions are not still clearly resolved.

Therefore, we paid our attention in part to this important topic as well. We used optogenetic approach, i.e., photo-stimulation of inhibitory dorsal horn neurons and patch-clamp recording in spinal cord slices from transgenic VGAT-ChR2-eYFP mice to study changes of inhibitory and excitatory neurotransmission in different pain models. We compared the changes occurring in the model of PIPN, peripheral inflammation and chronic constriction injury of the sciatic nerve.

Our preliminary data clearly show that in the different pain conditions tested, significant changes in spinal synaptic transmission occur. In more detail, our results show that PIPN, carrageenan-induced peripheral inflammation, and CCI of sciatic nerve changed the balance between the excitatory and inhibitory synaptic transmission in spinal cord dorsal horn excitatory neurons. These findings are in agreement with previously published studies (Ahmadi *et al.*, 2002; Moore *et al.*, 2002; Scholz *et al.*, 2005; Yan *et al.*, 2015a), and confirm the establishment of pathological changes in our models/conditions.

The sEPSC frequency was increased and the sIPSC frequency was significantly reduced in all the experimental models. Changes in the *frequency* of postsynaptic currents are usually attributed to altered properties of presynaptic terminals through mechanisms that regulate intracellular calcium concentration and vesicular release of neurotransmitters. However, the *amplitude* change could not be explained so easily and not necessarily must be related to postsynaptic mechanisms. For example, the insertion or internalization of receptors (e.g., AMPA receptors during LTP/LTD) into/from postsynaptic plasmatic membrane may cause bigger or smaller postsynaptic currents with the same quantal content in a synaptic vesicle (Latremoliere & Woolf, 2009). On the other hand, if the vesicle release is somehow affected, an increase or decrease of postsynaptic current amplitude could be produced by presynaptic mechanism due to changing of the quantal content.

Hence, significant changes both of sIPSC and sEPSC frequency in our preliminary unpublished results strongly suggest that presynaptic changes play a substantial role in the development of disinhibition in our models.

However, the interpretation of decreased le-IPSC is more complicated. Nevertheless, we did not find any changes in the kinetics of le-IPSC (such as in rise time, decay time or in *Tau*

constants), which suggests that properties of postsynaptic receptors are not significantly changed under our experimental conditions.

One of the possible explanations of decreased amplitudes of le-IPSC in our experiments is that the vesicular pool of GABA and glycine is reduced under pathological conditions. Therefore, the supramaximal stimulation could decrease the amplitudes of le-IPSCs compared with unaffected control, because the quantal content is reduced. One more explanation is that the release probability (p$_r$) of neurotransmitter release may be affected. Both of these explanations correlate with the findings that sIPSC and sEPSC frequency is changed, which also represent presynaptic mechanism.

Nevertheless, the presented data are preliminary and a more detailed experiment, such as paired-pulse ratio (PPR) stimulation is necessary to determine entirely the involvement of *pre-* or *postsynaptic mechanism* of disinhibition in our experimental models. Even, both pre- and postsynaptic mechanisms may occur in a different manner in pain states tested in our experiments.

14.3. Na$_v$1.7 Inhibition as a Potential Target to Alleviate Burn Injury-Induced Pain

Recently, it has been shown that Na$_v$1.7-mediated currents significantly contribute to the hyperexcitability of sensory neurons following burn injury (Shields *et al.*, 2012). Our electrophysiological experiment confirms the important role of Na$_v$1.7 in spinal nociceptive processing following partial thickness burn injury of scalding-type, which includes damage of epidermis and eventually upper parts of dermis as well.

We showed that the frequency of sEPSCs was significantly increased when the spinal cord slices were prepared one hour after burn injury. This increase of sEPSCs frequency was significantly reduced by the application of tarantula venom-peptide ProTxII, an antagonist of Na$_v$1.7. The differential effect of ProTxII in slices prepared from sham and burn-injured animals could be due to the minor role of Na$_v$1.7 under control conditions, whereas it gains a much more important role in generating spontaneous activity of nociceptive primary sensory neurons after burn injury. It could be mediated due to neuroinflammatory processes that affect Na$_v$1.7 on the central terminals in the spinal cord after burn injury. Increased expression of Na$_v$1.7 also could play the role in this increased spontaneous activity of nociceptive afferents, but probably in later time points.

Using capsaicin test at the end of each sEPSCs recording we confirmed that all the tested neurons responded to capsaicin. This strongly suggests that all of the tested neurons are involved in the processing of nociceptive information.

Our collaborators from Imperial College London performed western blot analysis of Na$_v$1.7 protein expression from ipsi- and contralateral L4–L5 DRGs of rats five minutes and three

hours after the induction of the burn injury. It has been shown that burn injury significantly upregulates $Na_v1.7$ protein expression in ipsilateral DRGs of rats three hours after burn injury. No changes in $Na_v1.7$ protein expression has been found five minutes after injury. Nevertheless, quantification of $Na_v1.7$ immunostaining in L4–L5 DRG confirms upregulation of $Na_v1.7$ expression at 180 min after burn injury as well as western blot analysis (Torres-Perez *et al.*, 2018). Similar up-regulation has been reported previously in other peripheral inflammatory models (Black *et al.*, 2004; Gould *et al.*, 1998). Shields *et al.* reported the increased $Na_v1.7$ protein expression and the increased $Na_v1.7$-mediated current density 3 hours and 2 days after the burn injury, which support the importance of $Na_v1.7$ contribution to pain hypersensitivity during the entire course of burn injury (Shields *et al.*, 2012).

Burn injury induced a biphasic upregulation in the expression of a phosphorylated form of transcription factor CREB (cyclic adenosine monophosphate (cAMP) response element-binding protein; p-CREB; Torres-Perez *et al.*, 2018). p-CREB is a common downstream effector of various signaling pathways implicated in the regulation of transcriptional changes associated with a use-dependent increase in the activity and excitability (sensitization) of primary sensory neurons by noxious stimuli (Qiao & Vizzard, 2004). The first peak of biphasic upregulation has been found after 5 minutes and it could be due to the activation of sensory neurons by excessive heat and/or by mediators released from damaged/degenerating cells (Torres-Perez *et al.*, 2018). The second increase occurs after 3 hours and it could be possibly due to the activation of neurons by inflammatory mediators. Importantly, double immunostaining of p-CREB and $Na_v1.7$ revealed a high degree of co-expression after burn injury, which confirmed that $Na_v1.7$ expressing DRG neurons are activated following burn injury (Torres-Perez *et al.*, 2018).

The following aim of our study was devoted to comparing the efficacy of morphine—the drug currently used to control pain, with a ProTxII on spinal nociceptive processing. Expression of two markers of nociceptive activation of dorsal horn neurons—the phosphorylated form of ERK1/2 (p-ERK1/2), and phosphorylated serine 10 in histone H3 (p-S10H3) was analyzed. While p-ERK1/2 is well established as a marker of nociceptive activation (Ji *et al.*, 2002; White *et al.*, 2011), p-S10H3 is a novel marker for nociceptive activation of spinal dorsal horn neurons (Torres-Perez *et al.*, 2017). Intraperitoneal administration of both morphine and ProTxII, which respectively activates μ-opioid receptors and inhibits $Na_v1.7$, significantly reduced the burn injury-induced upregulation of both p-ERK1/2 and p-S10H3 expression in dorsal horn neurons (Torres-Perez *et al.*, 2018).

These results are in full agreement with previous findings that morphine reduces spinal nociceptive processing (Yaksh, 1981). The effect of ProTxII is in contrast with some findings when intravenous or intraperitoneal injection of ProTxII does not reduce pain-related behavior. The lack of ProTxII-mediated effect after *i.v.* or *i.p.* administration on pain-related behavior was attributed to the inability of the ProTxII to access $Na_v1.7$ in intact peripheral nerves and to pass through the BBB (Schmalhofer *et al.*, 2008). Nevertheless, the analgesic effect of ProTxII was currently reported, e.g., ProTxII (*i.t.*) dose-dependently prevented, and reversed PAC-induced

mechanical hypersensitivity and reduce spontaneous action potential firing in DRG neurons (Li *et al.*, 2018).

In addition to those findings, Torres-Perez *et al.* (2018) suggest that ProTxII may also reach $Na_v1.7$ following *intraperitoneal injection*. It is suggested based on previous findings, that the ProTxII-mediated inhibitory effect on spinal nociceptive processing could be due to ProTxII-induced inhibition of $Na_v1.7$ expressed on free nerve endings at the injured tissues as well as on the $Na_v1.7$-expressing primary sensory neurons. DRGs are less protected by the BBB, in comparison with CNS (Allen & Kiernan, 1994) and therefore are DRG neurons potentially more susceptible to effect of intraperitoneally administered compounds, such as ProTxII in our study.

In conclusion, we showed that ProTxII significantly reduced aberrant activity induced by partial thickens burn injury in the population of capsaicin-sensitive nociceptive spinal cord dorsal horn neurons in the rat. $Na_v1.7$ inhibition by ProTxII represents a potentially promising therapeutic approach that could produce a significant analgesics effect with significantly fewer side-effects than opioids (Torres-Perez *et al.*, 2018).

14.4. Peripheral Inflammation Affect the Modulatory Effect of Anandamide Precursor 20:4-NAPE

Endocannabinoids, such as anandamide (AEA), play an important role in modulating spinal nociceptive processing and may significantly affect the development of pain. We showed in our experiments that application of the substrate for AEA synthesis—20:4-NAPE (NAPE), increased AEA concentration *in vitro*. Although direct effects of NAPE or indirect effects of others NAPE/AEA-related metabolites on some other receptors cannot be categorically excluded, we propose that the great majority of the effects induced by NAPE as reported in our study, was mediated through the synthesis of AEA acting on CB_1 receptors and TRPV1 channels (Nerandžič *et al.*, 2017). This assumption is in agreement with previous findings (Zygmunt *et al.*, 1999).

The use of NAPE, instead of AEA directly, has allowed us a distinctive opportunity to study the role of the spinal endocannabinoid system, because in these settings physiological mechanisms of anandamide synthesis played an important role (including the level of their activity and local distribution, which may alter the local concentration of anandamide). It is more likely that other receptors and biological pathways would have been activated if AEA was applied to the sample directly. This method of local endogenous AEA production "on demand" from its precursor NAPE may prove to be of advantage also in the clinical settings for pain treatment.

In our electrophysiological experiment, we compared the effect of NAPE in control conditions and in animals with acute peripheral inflammation induced by injection of 3% carrageenan into both hind paws. While NAPE treatment inhibited the excitatory synaptic transmission (sEPSC and eEPSC) in both naive and inflammatory conditions, acute peripheral inflammation

altered the underlying mechanisms of NAPE (respectively of AEA action) at the spinal cord level in the rat.

The NAPE-induced attenuation of the frequency of sEPSCs, respectively amplitude of eEPSCs, was mediated mainly by activation of presynaptic CB_1 receptors under normal conditions. Although CB_1 receptors could be located post-synaptically, most studies suggest an exclusive presynaptic location, where its activation leads to reduced release of neurotransmitters (Liang *et al.*, 2004; Morisset & Urban, 2001; Nyilas *et al.*, 2009; Pernia-Andrade *et al.*, 2009; Veress *et al.*, 2013). In our experimental conditions, the inhibitory synaptic transmission was pharmacologically blocked using bicuculline and strychnine. Therefore, we can expect that the inhibitory effect of NAPE is mediated mainly by attenuation of excitatory neurotransmitter release from central endings of CB_1-expressing primary afferent neurons.

Under control conditions, the effect of NAPE was prevented by application of CB_1 receptor antagonist PF514273. Pretreatment with TRPV1 antagonist SB366791 did not affect the NAPE mediated effect on sEPSCs in control conditions. Pretreatment with either PF514273 or SB366791 alone did not affect significantly the sEPSCs frequency (Nerandžič *et al.*, 2017). In spinal cord slices prepared after the induction of acute peripheral inflammation, NAPE application induced a significant decrease of sEPSCs frequency/eEPSC amplitude, similarly to observation in naive control animals. However, under inflammatory conditions, just SB366791 alone reduced the sEPSCc frequency, which suggests tonic activation of presynaptic TRPV1 channels. The application of CB_1 blocker alone did not change frequency significantly under inflammatory conditions, but nevertheless, there is a shift to higher frequency compared with naive control. More interestingly, co-application of CB_1 blocker with NAPE leads, in contrast to control conditions, to the significant increase in sEPSCc frequency compared to the effect of NAPE alone (Nerandžič *et al.*, 2017).

These results indicate that under inflammatory conditions, the inhibitory effect of NAPE is still CB_1-mediated, while the potentiating effect of NAPE on sEPSCs is mediated via TRPV1 channels. Interestingly, this TRPV1-mediated potentiating effect is unmasked only when CB_1 receptors were blocked.

Therefore, it seems that preferentially inhibitory CB_1-mediated effect of NAPE is under inflammatory conditions partly modified by an additional TRPV1-dependent mechanism— probably because of sensitization of TRPV1 by inflammatory mediators.

Data from eEPSCs experiments showed slight, but not significant attenuation of NAPE-mediated effect during inflammation. In naive animals, acute NAPE application reduces eEPSCs amplitudes to 70.5 ± 9.0 %, whereas in slices prepared from inflammatory animals, the effect of NAPE was slighter 78.5 ± 6.6 %, but still significant (Nerandžič *et al.*, 2017). However, in contrast to sEPSCs, the amplitude of eEPSCs was not increased significantly following coapplication of CB_1 blocker and NAPE, when sensitized TRPV1 were "uncovered". This observation is in good agreement with previous findings showing that the neurotransmitter release induced by action potentials by dorsal root stimulation may be blocked by activation of TRPV1 channels

(Baccei *et al.*, 2003; Yang *et al.*, 1999). Thus, it is possible that activation of TRPV1 on presynaptic terminals of DRG neurons by NAPE/AEA reduced the action potential-dependent glutamate release from central endings of primary afferents and thus contributed to the decrease of eEPSCs amplitude in the postsynaptic neuron. This mechanism could contribute, at least in part, to an analgesic action of AEA under inflammatory conditions.

In conclusion, our data indicate that application of exogenous NAPE induced inhibitory effects is mediated mainly by CB_1 receptors in naive animals, while TRPV1-mediated mechanisms were also involved after acute peripheral inflammation (Nerandžič *et al.*, 2017). We also suggest that applying of AEA substrate for its local synthesis may be more effective for analgesic purposes than systemic anandamide application or inhibition of its degradation (Mallet *et al.*, 2016).

15. Conclusion

Pain is usually induced following exposure to different stimuli of noxious intensity. Although acute nociceptive pain is accompanied by an unpleasant sensation, it represents an important physiological mechanism, which helps protect our bodies from harmful and damaging stimuli.

However, pain is also a common annoying symptom of many clinical syndromes and diseases, when it loses its protecting function and cause the suffering of patients. In particular, the treatment of chronic and neuropathic pain represents a serious issue because currently available analgesia is ineffective, inappropriate or it has adverse effects in many cases.

In the dorsal horn of the spinal cord is located the first synapse of the pain pathway. Dorsal horn represents a major modulatory site of the nociceptive pathway. Nociceptive synaptic transmission in the dorsal horn can be substantially modulated by local interneurons, descending pathways and by pronociceptive mediators, which are released from both neurons and non-neuronal cells. Modulation may significantly influence the nociceptive signaling and subsequently pain perception, especially under different pathological conditions. Therefore, we have focused on the study of modulatory mechanisms that occur in the spinal dorsal horn.

Despite the huge increase in the number of published studies in the field of pain during the last two decades, management of chronic and neuropathic pain still represents a serious problem. While many of modulatory mechanisms have been already identified and described, many other mechanisms remain unrecognized.

A PubMed search using the keyword *"pain"* revealed that in total 194.657 publications have been published in years 1999–2008, whereas almost twofold increase in the number of publications was found in next decade (354.292 items, years 2009–2018). Figure 15.1 (p. 130) illustrates a detailed analysis of the number of publications searched using different „keywords" related to the topics of this thesis. A substantial increase is especially in publications focused on *"neuroinflammation and pain"*, *"Na_v1.7 and pain"* and *"TLR4 and pain"*. An almost twofold increase is also in publications focused on *"paclitaxel and neuropathy"*. Figure 15.1 also shows a noteworthy increase in the number of publications on *"capsaicin receptor or TRPV1 and pain"* since the year 2003, which is followed by an actual steady state. The decline in interest in TRPV1 is probably due to the failure of the first generation TRPV1-targeting drugs in clinical trials. Relatively little attention is given to the role of *"disinhibition and pain"* during the whole period, despite the importance of disinhibition in the development of different pain states.

Hence, the aim of our experiments was to explore some new mechanisms and potential therapeutic approaches on how to better manage pain states, in which currently used analgesics are ineffective or inappropriate.
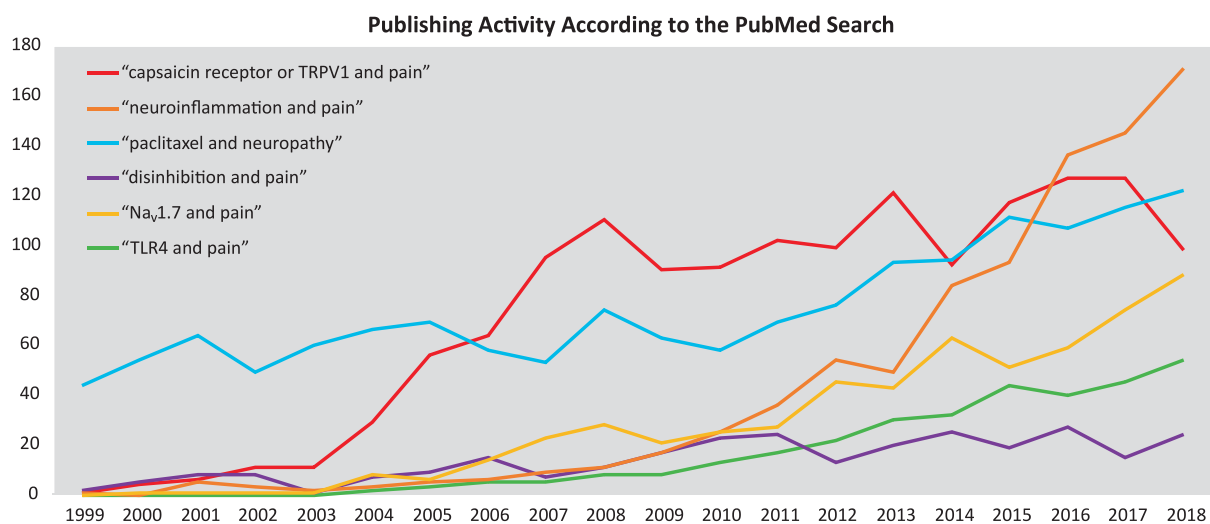
Figure 15.1.: **A PubMed search of publishing activities in the last two decades.** The graph shows the number of publications searched using different "keywords" (see legend) related to the topics of this thesis. A substantial increase reveals also search using the keyword "pain". In total 194.657 publication have been published in years 1999–2008, and actually 354.292 items have been published in the next decade, in years 2009–2018.

In this doctoral thesis are presented results from five original articles and also unpublished results, all focused on mechanisms of modulation of nociceptive synaptic transmission under pathological conditions.

In the model of paclitaxel-induced peripheral neuropathy, we showed for the first time that the direct functional interaction between TLR4 and TRPV1 receptors play important role in neuronal activation, sensitization, and behavioral hypersensitivity in PAC-induced CIPN (Li *et al.*, 2015a). In our following study, we have demonstrated that TRPV1-dependent mechanism is necessary to PAC-induced enhancement of c-Fos protein expression in the dorsal horn neurons (Kalynovska *et al.*, 2017). Moreover, we currently reported that PI3K plays an important role (*I.*) in the PAC-induced mechanical allodynia, and (*II.*) in the modulation of TRPV1 sensitivity and tachyphylaxis of capsaicin-evoked responses (Adámek *et al.*, 2019).

Our electrophysiological experiment also confirms the important role of $Na_v1.7$ in spinal nociceptive processing following partial thickness burn injury of scalding-type. We showed that $Na_v1.7$ inhibitor ProTxII significantly reduced aberrant activity induced by burn injury in the population of capsaicin-sensitive nociceptive spinal cord dorsal horn neurons in the rat model of burn-injury (Torres-Perez *et al.*, 2018).

We also reported the promising analgesics effect of 20:4-NAPE, which seems to be a potentially good source for endogenous AEA synthesis, Moreover, our data indicate that NAPE-induced inhibitory effects were mediated mainly by CB_1 receptors in naive animals, while TRPV1-mediated mechanisms were also involved after acute peripheral inflammation (Nerandžič *et al.*, 2017).

Finally, our preliminary data strongly suggest that disinhibition significantly contributes to the development of mechanical allodynia and to the changes in synaptic transmission in the dorsal horn in the model of PIPN, CCI and following acute peripheral inflammation.

Taking together, these data well demonstrates that nociceptive synaptic transmission is substantially influenced under pathological conditions, and that appropriate intervention and pharmacological treatment can help alleviate increased nociceptive transmission or pain-related behavior in animals.

I hope that the results presented in this doctoral thesis represent a small step in the process of a deeper understanding of these complicated processes of modulation of spinal nociceptive transmission. However, a lot of work is still ahead of us to fully understand these complex processes and to develop new and better pain therapy.

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Appendix

List of Publications Related to the Doctoral Thesis:

P. Adámek, M. Heleš and J. Paleček, **2019**, Mechanical allodynia and enhanced responses to capsaicin are mediated by PI3K in a paclitaxel model of peripheral neuropathy: *Neuropharmacology*, v. 146, p. 163–174.

(**IF = 4.249**, Quartile 1, ***Times Cited: 0*** *4/2019 from WoS)*

Torres-Perez, J. V., **P. Adámek**, J. Paleček, M. Vizcaychipi, I. Nagy, and A. Varga, **2018**, The Na$_v$1.7 blocker protoxin II reduces burn injury-induced spinal nociceptive processing: *Journal of Molecular Medicine (Berl).* v. 96(1), p. 75–84.

(**IF = 4.938**, Quartile 1, ***Times Cited: 1*** *4/2019 from WoS)*

Nerandžič, V., P. Mrózková, **P. Adámek**, D. Špicarová, I. Nagy, and J. Paleček, **2018**, Peripheral inflammation affects modulation of nociceptive synaptic transmission in the spinal cord induced by *N*-arachidonoylphosphatidylethanolamine: *British Journal of Pharmacology*, v. 175(12), p. 2322–2336.

(**IF = 6.81**, Quartile 1, ***Times Cited: 2*** *4/2019 from WoS)*

Kalynovska, N., **P. Adámek**, and J. Paleček, **2017**, TRPV1 receptors contribute to paclitaxel-induced c-Fos expression in spinal cord dorsal horn neurons: Physiological Research, v. 66(3), p. 549–552.

(**IF = 1.324**, Quartile 4, ***Times Cited: 3*** *4/2019 from WoS)*

Li, Y., **P. Adámek**, H. Zhang, C. E. Tatsui, L. D. Rhines, P. Mrózková, Q. Li, A. K. Kosturakis, R. M. Cassidy, D. S. Harrison, J. P. Cata, K. Sapire, H. Zhang, R. M. Kennamer-Chapman, A. B. Jawad, A. Ghetti, J. Yan, J. Paleček, and P. M. Dougherty, **2015**, The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4: *Journal of Neuroscience*, v. 35(39), p. 13487–13500.

(**IF = 5.917** , Quartile 1, ***Times Cited: 57*** *4/2019 from WoS)*