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The role of TRPV1 receptors in nociceptive signalling at spinal cord level

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Declaration

This dissertation is submitted as fulfilment of doctoral studies at Faculty of Science, Charles University in Prague. I hereby confirm that this thesis was independently written by me. No material was used other than that referred to. Sources directly quoted including figures and photos, have been correctly denoted, those not otherwise indicated, belong to the author. No part of this thesis has been submitted by the author to obtain another academic degree.

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Acknowledgements

Abstract

Modulation of nociceptive synaptic transmission in the spinal cord dorsal horn plays a key role in the development and maintenance of pathological pain states and chronic pain diseases. Important role in this process play transient receptor potential vanilloid 1 receptors (TRPV1), present on presynaptic endings of primary afferents in the superficial spinal cord dorsal horn. Changes in TRPV1 activity have significant impact on nociceptive transmission. There are number of processes that influence the function of spinal TRPV1 receptors. This work is focused on the role of protease-activated receptors type 2 (PAR2), C-C motif chemokine ligand 2 (CCL2) and the effect of chemotherapeutic drug paclitaxel in modulation of synaptic nociceptive transmission and activation of TRPV1 receptors.

PAR2 receptors belong to a family of four G-protein-coupled receptors activated by proteases. The role of PAR2 receptors in pain perception is closely related to their presence in a population of dorsal root ganglion neurons, where they are also co-expressed with TRPV1. Activation of PAR2 may lead to peripheral and central sensitization. Chemokine CCL2 and its main receptor CCR2 were suggested to be an important factor in the development of neuropathic pain after peripheral nerve injury. In our study we focused on the effect of CCL2 application on TRPV1 receptor activation and nociceptive signalling. Paclitaxel is an antitumor drug which clinical use is limited by the appearance of neuropathic pain conditions. The aim of our study was to investigate paclitaxel effect on presynaptic TRPV1 receptors in the spinal cord dorsal horn.

Experiments in this thesis were preferentially aimed to study the role of PAR2 receptors in nociceptive processing and modulation of synaptic transmission, using behavioural and electrophysiological techniques. We showed that intrathecal application of PAR2 activating peptide SLIGKV-NH$_2$ caused hyperalgesia in naïve animals that was prevented by pre-treatment with TRPV1 antagonist SB 366791 and protein kinases inhibitor Staurosporine. Patch-clamp recordings of post synaptic excitatory currents from superficial dorsal horn neurons in acute spinal cord slices was used to demonstrate that activation of PAR2 receptors by SLIGKV-NH$_2$ caused a decrease in the frequency of mEPSC, but increased the frequency of sEPSC and also increased the amplitude of dorsal root stimulation evoked EPSC. These effects were also significantly attenuated by application of SB 366791 and staurosporine. Our results suggest that presynaptic PAR2 receptors may play an important role in the modulation of nociceptive synaptic transmission in the spinal cord dorsal horn.

Results of another study demonstrated that changes induced by CCL2 application were largely mediated through activation of TRPV1 receptors, suggesting importance of PAR2 receptors in pain modulation. Paclitaxel application induced increased frequency of mEPSC currents that was prevented by TRPV1 receptors antagonist, implying their contribution to paclitaxel-induced acute and chronic neuropathic pain.

Our results demonstrate an important role of spinal TRPV1 receptors in modulation of nociceptive transmission in the spinal cord dorsal horn. We have shown that activation of PAR2, application of CCL2 and paclitaxel induced changes which lead to modulation of synaptic transmission mediated primarily by TRPV1 receptors. This further confirms the fundamental role of TRPV1 receptors in pain modulation at spinal cord level and supports their importance in pathological pain states.

Abstrakt

Modulace nociceptivního synaptického přenosu v zadním rohu míšním hraje klíčovou úlohu v rozvoji a udržování zejména patologických a chronických bolestivých stavů. Důležitou roli v tomto procesu hrají vaniloidní receptory typu 1 (TRPV1), které jsou přítomny na presynaptických zakončeních primárních aferentních neuronů v superficiálních oblastech zadního rohu míšního. Změny v aktivitě míšních TRPV1 receptorů mají velký dopad na nociceptivní přenos. Existuje celá řada procesů, jejichž účinek na nociceptivní přenos je zprostředkován TRPV1 receptory a které ovlivňují jejich funkci. Tato práce se zaměřila zejména na úlohu proteázou aktivovaných receptorů typu 2 (PAR2), chemokinu CCL2 a chemoterapeutika paclitaxelu, spojeného s aktivací TLR4 receptorů.

PAR2 patří do rodiny receptorů spojených s G proteiny a jsou aktivovány proteázami. Role PAR2 receptorů při vnímání bolesti je úzce spjata s jejich přítomností v populaci neuronů míšních ganglii kde jsou často exprimovány s TRPV1 receptory. Tato práce se proto soustředila na modulaci nociceptivní signalizace po aktivaci míšních PAR2 receptorů. Chemokin CCL2 se ukázal být důležitým faktorem v rozvoji neuropatické bolesti po poranění nervu. V provedených experimentech byl účinek aplikace CCL2 studován hlavně vzhledem k aktivaci míšních TRPV1 receptorů. Paclitaxel je protinádorový lék, jehož klinické využití je limitováno vznikem neuropatických bolestivých stavů. Cílem naší práce bylo studovat jeho vliv na TRPV1 a TLR4 receptory.

Role míšních PAR2 receptorů byla studována za použití behaviorálních a elektrofyziologických technik. Prokázali jsme, že po intrathekálním podání PAR2 aktivačního peptidu SLIGKV-NH$_2$ dojde k rozvoji tepelné hyperalgesie, která je blokována podáním TRPV1 antagonisty SB 366791 nebo inhibitoru proteinkináz Staurosporinu. Snímání postsynaptických excitačních proudů ze superficiálních neuronů zadního rohu míšního ukázalo, že aplikace PAR2 aktivačního peptidu vyvolá snížení frekvence mEPSC, ale naopak nárůst frekvence sEPSC i amplitudy u evokovaných EPSC. Tyto účinky byly také významně zeslabeny aplikací SB 366791 a staurosporinu. Presynaptické PAR2 receptory mohou hrát důležitou roli v modulaci nociceptivního synaptického přenosu v zadním rohu míchy.

Další výsledky ukázaly že CCL2 také působí přes aktivaci míšních TRPV1 a že tato aktivace hraje důležitou roli v modulaci nociceptivní signalizace. Dále bylo zjištěno, že aktivace TLR4 paclitaxelem vede k senzitizaci centrálních TRPV1 receptorů, což může přispívat k paclitaxelem vyvolané akutní a chronické neuropatii.

Naše výsledky potvrdily důležitost míšních TRPV1 receptorů v modulaci nociceptivního přenosu v superficiální oblasti zadního rohu míchy. Ukázali jsme, že aktivace PAR2 receptorů a aplikace CCL2 nebo paclitaxelu v míše vede k modulaci synaptického nociceptivního přenosu zprostředkovaného zejména TRPV1 receptory. Tato aktivace a modulace TRPV1 receptorů může být důležitá zejména za patologických bolestivých stavů. Poznání funkce míšních TRPV1 receptorů může přispět k vývoji nových analgetik pro dosud velmi obtížně léčitelné stavy neuropatické bolesti.

List of abbreviations

$[Ca^{2+}]i$	Ca^{2+} intracellular Ca^{2+} concentration
2-APB	2-aminoethoxydiphenyl borate
5-HT	5-hydroxytryptamine
20-HETE	20-Hydroxyeicosatetraenoic acid
AA	amino acid
AC	adenylate cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	glutamatergic AMPA receptor
ANOVA	analysis of variance
ATP	adenosine triphosphate
BK	large-conductance calcium- and voltage-activated potassium channels
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumine
Ca^{2+}	calcium ion/s
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin dependent kinase II
cAMP	cyclic adenosine monophosphate
CC – CXC – CX3C	chemokine families
CCD	chronic constriction of the DRG
CCI	chronic constriction injury
CCL2	chemokine CCL2
CCR2	chemokine CCL2 receptor
$CCR2^{-/-}$	CCR2 knock out
CFA	Freund's complete adjuvant
CGRP	calcitonin-gene related peptide
CIPN	chemo-induced peripheral neuropathy
CNS	central nervous system
COX	cyglogen oxygenasa
DAG	diacyl glycerol
Damps	damage (danger)-associated molecular patterns
DIC	differential interference contract
DH	dorsal horn
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EAA	excitatory amino acid
ECS	extracelullar solution
eEPSC	evoked excitatory postsynaptic current
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPSC	excitatory postsynaptic current
ERK	extracellular signal-regulated kinase
Fos	proto-oncogene protein
GABA	gamma-Aminobutyric acid
GDNF	glial cell-line derived neurotrophic factor
GluR	glutamate receptor subunit

GPCRs	G protein–coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HT	high threshold
IGF-1	insuline-like growth factor 1
IL-1β	interleukin-1β interleukin-1β
IP3	inositol trisphosphate
IPSC	inhibitory postsynaptic current
LPS	lipopolysaccharide
LT	low threshold
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MD-2	lymphocyte antigen 96
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamate receptor
mRNA	messenger Ribonucleic acid
Na$^+$	natrium cation
NADA	N-arachidonoyl-dopamine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth facror
NK1	neurokinin
NK$_1$R	neurokinin receptor 1
NMDA	N-methyl-D-aspartate
NMDAR	glutamatergic NMDA receptor
NO	nitric oxide
NR	NMDAR subunit
OLDA	N-oleoyl-dopamine
P2X3	purinergic receptors
P2Y1	purinergic receptors
p38	p38 mitogen-activated protein kinases
PAF	primary afferent fibre
Pamps	pathogen-associated molecular patterns
PAR	protease activated receptor
PAR2	protease activated receptor type 2
pERK	phosphorylated extracellular signal-regulated kinase
PG	prostaglandin
PGE$_2$	prostaglandin E2
pH	potential of hydrogen
PI3K	phosphatidylinositol-3 kinase
PI4K	phosphatidylinositol-4 kinase
PIP$_2$	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PLA$_2$	phospholipase A2
PLC	phospholipase C
PNS	peripheral nervous system
PSNL	partial sciatic nerve ligation
PWL	paw withdrawal latency

PWT	paw withdrawal threshold
Raf1	proto-oncogene, serine/threonine kinase
RT-PCR	reverse transcription polymerase chain reaction
RTX	resiniferatoxin
S1-6	subunit
SEM	standard error of mean
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
SNI	spared nerve injury model
SNL	spinal nerve ligation
SP	substance P
TLRs	Toll-like receptors
TLR4	Toll-like receptor 4
TNFα	tumour necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
TrkA	nerve growth factor receptor
TRP	transient receptor potential
TRPA	transient receptor potential Ankyrin family
TRPM	transient receptor potential Melastatin family
TRPV1	transient receptor potential Vanilloid 1
TRPV1$^{-/-}$	TRPV1 knock out
TTX	tetrodotoxin
VGCC	voltage-dependent calcium channels
N-, P/Q-, L-, R- T-	neural, purkinje, long-lasting, residual, transient type of VGCC
WDR	wide dynamic range neuron

TABLE OF CONTENTS

1. INTRODUCTION

The ability to provide information about the occurrence of tissue damage is one of the vital functions of the nervous system. **By the International Association for the Study of Pain (IASP) definition, pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage".** The sensation of pain has evolved to protect tissues against severe injury and disruption of the organism integrity and contributes to the surviving of the organism. It helps each individual to avoid situations that are likely to be damaging. Every individual perceives pain in a specific way; the same stimulus can trigger various levels of pain in different individuals. Pain perception depends on the experience and actual emotional and physical condition. Peripheral nervous system responds to noxious stimuli and provides alerts for the organism to avoid potential injury. The entire process, from detection in the peripheral tissues via processing in spinal cord and in the brain, is called nociception. But nociceptive signalling does not necessarily result in a sensation of pain. Only after nociceptive perception is processed in various centres of the brain, then we may perceive pain.

Pain is also the prime symptom of many diseases and warns the organism that something is damaged or does not function properly. Pain, however, may fail to fulfil its protective function, and become a disease itself. Nociceptive signalling dysfunction can occur at any level of the nervous system and the result is a pathological pain. Pathological pain unlike physiological pain has a very negative influence on the organism, it is difficult to cure and leads to reduced quality of life. Nowadays by any measure, pain is still a significant health problem. Globally, it has been estimated that 1 in 10 adults suffers from chronic pain. In order to establish new effective methods of treatment for different types of pain, detailed understanding of the nociceptive signalling mechanisms is needed. Especially the changes that occur under pathological conditions are essential for understanding the underlying processes during pain manifestation.

Like other sensory modalities, the potentially damaging stimuli are usually detected by specific receptors – nociceptors. Unlike most of other receptors, nociceptors do not respond till to very intense stimulus, and they do not show adaptation to a continuing stimulus, on the contrary, they may become more sensitive. The information from the peripheral nociceptors is further transmitted in

the form of action potential into the spinal cord and then into the brain. First synapse in dorsal horn of spinal cord is important for modulation of nociceptive transmission. Synaptic transmission depends on release of other transmitters and neuromodulators and the activation of many different receptors and channels. Important factor is also phosphorylation of receptors that regulates their biophysical properties and cellular localization.

Spinal **Transient receptor potential Vanilloid 1 (TRPV1)** receptors were shown to have an important role in modulation of nociceptive transmission, especially under pathological conditions (Lappin et al., 2006, Spicarova and Palecek, 2008, Chen et al., 2009, Spicarova and Palecek, 2009). It was suggested that under these conditions, they become phosphorylated and increase their sensitivity to endogenous agonists (Spicarova and Palecek, 2008, 2009, 2010). There are number of processes that influence the function of spinal TRPV1 receptors present on the presynaptic endings of primary afferents in the superficial spinal cord dorsal horn.

In this thesis we have focused on the role of **Protease-activated receptor type II (PAR2)**, **C-C chemokine receptor type 2 (CCR2)** and **Toll-like receptor 4 (TLR4)** that are co-expressed with TRPV1 in the DRG neurons and may influence their activity**.**

PAR2 belongs to a family of G-protein-coupled receptors and they are cleaved and activated by proteases (Vergnolle, 2000, Ossovskaya and Bunnett, 2004). PAR2 are involved in response to tissue injury, protease-driven inflammation, nociception and also in tissue repair. Within the CNS, the endogenous activators for PAR2 and the physiological significance of its activation remain unclear, but potential candidates include tryptase, trypsinogen IV and neurotrypsin (Bushell, 2007). A large number of DRG neurons express PAR2 together with TRPV1 (Steinhoff et al., 2000, Amadesi et al., 2004). PAR2 are also present in the spinal cord dorsal horn. The role of PAR2 in spinal nociceptive signalling modulation was the main subject of my experiments summarized in this dissertation. Detailed knowledge of spinal PAR2 and their signalling, in particular their interaction with TRPV1, may contribute to more effective new approaches in therapy of pain.

CCR2 is the main receptor for **chemokine (C-C motif) ligand 2** (CCL2). The importance of **CCL2**, was established especially in the process of neuropathic pain development after peripheral nerve injury which triggered CCL2 release from

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the central endings of primary nociceptive neurons and attenuated pain (Gao et al., 2009b). CCL2 was shown to be present in predominantly small and medium size DRG neurons, co-expressed with substance P (SP), calcitonin gene-related peptide (CGRP) and TRPV1 receptors (Dansereau et al., 2008). In our experiments we have studied the effect of CCL2 application and TRPV1 activation on nociceptive signalling and modulation of synaptic transmission.

TLR4 receptors are responsible for activating innate immune system and are best known for recognizing lipopolysaccharide (LPS) and other ligands. TLRs are involved in pain transmission at the spinal cord level, especially in the transition of inflammatory pain to chronic status and in promoting the generation of nerve injury pain (Christianson et al., 2011). Stimulation of TLR4 initiates a series of signalling cascades that result in the activation of nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs) (Stokes et al., 2013). In our experiments we have studied activation of TLR4 and TRPV1 receptors by chemotherapeutic drug paclitaxel in a model of peripheral neuropathy.

Our results demonstrate an important role of spinal TRPV1 receptors in modulation of nociceptive transmission in the spinal cord dorsal horn under different conditions. We have shown the importance of PAR2 activation for pain modulation at the spinal cord level. Presented results also suggest that changes in nociceptive synaptic transmission after application of CCL2 and paclitaxel are mediated primarily by modulation of TRPV1 receptors function. This further confirms the fundamental role of TRPV1 receptors in pain modulation at spinal cord level and may contribute to more effective approaches in pain therapy.

2. STATE OF ART

2.1 Classification of pain

Pain can serve different purposes. One of them is an early-warning physiological protective system, essential to detect and minimize contact with damaging or noxious stimuli - nociceptive pain. The second type of pain is also adaptive and protective. By enhancing sensory sensitivity after unavoidable tissue damage, this pain support in healing of the injured body part by creating a situation that discourages physical contact and movement (Wall et al., 2006). Pain hypersensitivity, reduces further risk of damage and promotes recovery, as after an inflamed joint or in surgical wound, where normally innocuous stimuli now elicit pain. And then there is pathological pain that is not protective but maladaptive, resulting from abnormal functioning of the nervous system. This pathological pain which is not a symptom of any disorder but rather a disease state of the nervous system, can occur after a damage to the nervous system (neuropathic pain), but also in situations when there is no such damage or inflammation (dysfunctional pain). Conditions that evoke dysfunctional pain include fibromyalgia, irritable bowel syndrome, tension type headache, interstitial cystitis, and other syndromes that are characterized by substantial pain but no presence of a noxious stimulus and minimal peripheral inflammatory pathology (Woolf, 2010).

Pain is a subjective, psychological state that can only be described by verbal or magnitude scaling methods (Figure 1) (Breivik et al., 2008, Gelinas et al., 2008). The IASP also calls attention to the subjectivity of pain perception stating that if people consider their experience as pain and if they report it in the same ways as pain caused by tissue damage, it should be accepted as pain. Classifying pain is helpful to guide assessment and treatment. There are many ways to classify pain and different classifications may overlap.

Pain can be classified from many different points of view, temporal and physiological being the most common (Weiner, 2002; Wall et al., 2006).

Based on **different mechanisms** of development we distinguish between nociceptive, inflammatory and neuropathic pain. **Nociceptive pain** represents the normal response to a noxious insult or injury of tissues such as skin, muscles, visceral organs, joints or bones. Nociceptive pain is divided into somatic (superficial

or deep) or visceral. **Neuropathic pain** is initiated or caused by a primary lesion or disease in the somatosensory nervous system. **Inflammatory pain** is a result of activation and sensitization of the nociceptive pathway by a variety of mediators released at the site of tissue inflammation (Scholz and Woolf, 2002).
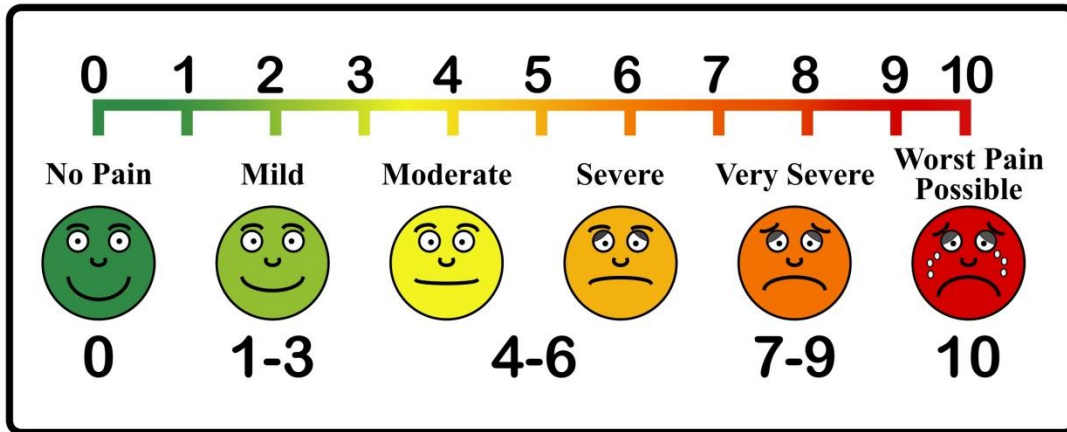
Figure 1. This pain assessment tool is intended to help patient care providers assess pain according to individual patient needs. Presents a 0-10 scale for patient self-assessment and use pictograms to express pain intensity. Adopted and adjusted from (https://openclipart.org/detail/218053/pain-scale, 26.1.2017)

According to the **duration** we usually distinguish acute, sub-chronic, and chronic pain. **Acute pain** is usually caused by direct stimulation of nociceptors due to damage to body tissue, a disease or a surgical intervention. Its duration is short-term. Subacute pain lasts longer – it is a matter of hours or days. After restoring the integrity of the organism it usually disappears. **Chronic pain** persists for a long time, even with no apparent cause, or is due to any cause disproportionately large. Generally, pain is considered as chronic pain when its last three to six months and longer (Millan, 1999).

Another possible criterion of division is **origin of pain**. Based on this criterion pain can be divided into somatic and visceral. Pain originating from the skin, muscles and the walls of body cavities (thoracic and abdominal wall) is considered somatic, while the visceral pain is induced in visceral organs in the body cavities (Cervero and Laird, 1999).

Pain is often characterized by a state of increased intensity of pain sensation induced by either noxious or ordinarily innocuous (non-noxious) stimulation of

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peripheral tissue (Sandkuhler, 2009). We are talking about allodynia and hyperalgesia (Figure 2).

Allodynia ("other pain") occurs when pain is felt after non-painful stimulus. Allodynia trigger pain response from stimuli which do not normally provoke pain. Thermal or mechanical stimuli can provoke allodynia, which may manifest as burning sensation and it often occurs after injury (Koltzenburg et al., 1992, Seal et al., 2009).

Hyperalgesia ("over pain") is an increased sensitivity to pain, which may be caused by damage to nociceptors, peripheral nerves or after central changes. It is characterised by increased pain sensation to painful stimuli. Temporary increased sensitivity to pain can also occur as part of sickness behaviour, the evolved response to infection (Sandkuhler, 2009, Clark et al., 2011).
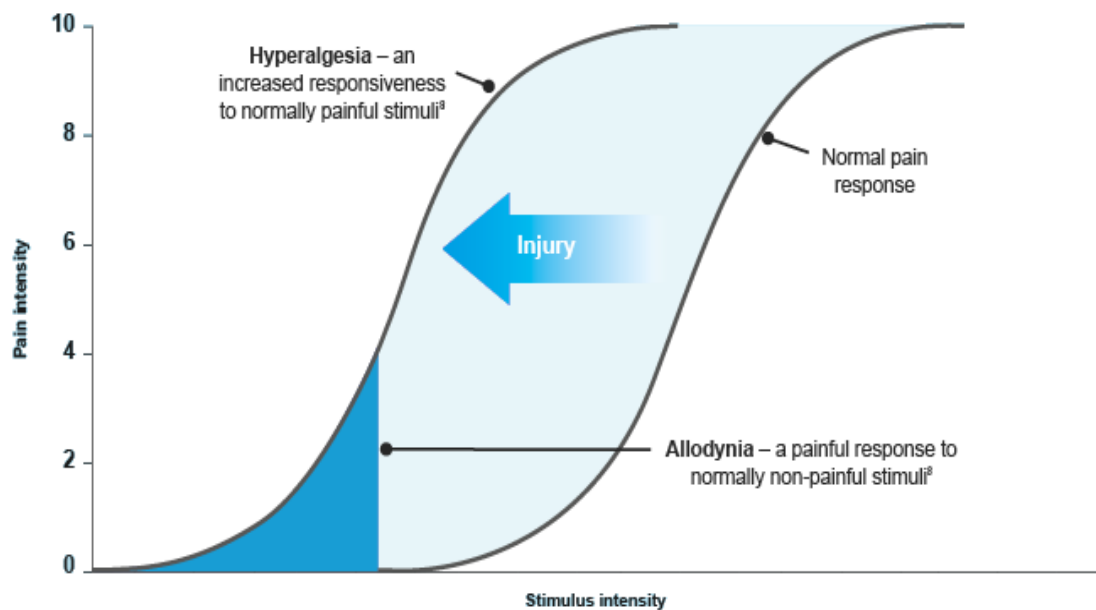
Figure 2. The normal pain response as a function of stimulus intensity is depicted by the curve at the right, where only strong stimuli are experienced as pain. However, a traumatic injury or a disease can shift the curve to the left. Then, noxious stimuli become more painful (hyperalgesia) and typically painless stimuli are experienced as pain (allodynia). Adopted from Textbook of Pain 2006 (Wall et al., 2006).

2.2 Anatomy and Physiology of Pain

Pain has much in common with other sensory modalities. Specific pain receptors on peripheral nerve endings are present in most body tissues, and respond only to damaging or potentially damaging stimuli. Subsequently these noxious stimuli are transmitted by specific nerve - fibres called primary afferent nociceptors through dorsal root ganglions (DRG) to the spinal cord. The primary afferent nociceptor contacts second-order neurons in the spinal cord. The spinal second-order neurons relay the message through ascending pathways to higher centres, including brain stem reticular formation, thalamus, somatosensory cortex, and limbic system. It is thought that the processes underlying pain perception involve primarily thalamus and cortex.

2.2.1 Detection and signalization of pain

In 1906, a hundred and ten years ago Sherrington assumed the existence of somatosensory neurons that are excited only in situation of tissue damage or injury. The sequence of nociceptive events, culminating in pain perception, starts with nociceptive stimulus activating nociceptors at the peripheral ending, of the first afferent neuron where the transduction into action potentials and encoding into trains of action potentials takes place (Kandel et al., 2000).

Nociception is a neural process that involves transducing mechanisms by which nociceptors detect and convey information about noxious stimuli to the CNS. Pain is usually felt when the nociceptors are irritated by damaging or potentially damaging stimulus, but it can be caused also by direct trauma of nociceptive fibers, or by injury in various areas of the CNS. Pathological pain may also arise independently from the stimulation of nociceptors, or incentives that are usually painless.

Electrophysiological studies have confirmed this hypothesis by identifying the primary sensory neurons that could be excited by stimuli inducing pain, such as high temperature, excessive pressure or chemical irritants, but conventional non-painful irritation did not cause their activation (Burgess and Perl, 1967). These primary nociceptive neurons are distinct from other sensory neurons and have higher specific threshold for activation. It was supposed, that acute pain is a sensory modality based on a similar principle as for example sight or smell. The mechanisms of transmission

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of the information applied to specific neurons receptive properties, which detect the quality or intensity of the stimulus (Julius and Basbaum, 2001). Stimuli from the external and internal environment are detected by peripheral projections of primary sensory neurons whose bodies are located in dorsal root ganglions. Neurons can respond to either one or more types of stimuli, and, accordingly they are divided into unimodal or polymodal (Julius and Basbaum, 2001).

Detection of a painful stimulus is carried through by specialized receptors - **nociceptors**. IASP defines nociceptors, as "high threshold sensory receptors of the peripheral nervous system, which are able to detect and transmit information about noxious stimuli "(IASP, 2011). **Nociceptor** - a sensory receptor that is capable of transducing and encoding noxious stimuli (Loeser and Treede, 2008). Nociceptors are peripheral endings of the primary afferent fibers of HD neurons (Aδ and C). C fibers are slowly conducting - velocity of signal transmission is 0.5 - 2.0 ms^{-1}, small diameter, unmyelinated fibers responsible for delayed, blunt and burning pain (Figure 3) (Millan, 1999). Aδ fibers are myelinated, fast-conducting - 12-30ms^{-1}, fibers are associated with experience of sharp pain (Millan, 1999). Although, non-nociceptive receptors may also respond to noxious stimuli, only nociceptors are capable of encoding the stimuli, with relevant properties like intensity and location (Woolf and Ma, 2007).

The cell bodies of **primary afferent nociceptors** are located in the **dorsal root ganglion (DRG)** and their central synapses lie mainly in superficial laminae of the spinal cord dorsal horn (Figure 3). Based on the responses to the peripheral stimuli, dorsal horn neurons involved in pain transmission are classified, as wide dynamic range responding to low and also high-intensity impulse as painful stimuli - **A fibers**, and high threshold nociceptive neurons responding to high intensity stimuli - **C fibres** (Wall et al., 2006). Low-threshold neurons respond to low intensity stimuli that under normal conditions are not perceive as painful (Basbaum et al., 2009). However, under pathological conditions, their activation by innocuous stimuli can be detected as painful. Molecular markers are often used for more detailed classification of primary sensory neurons. Small cells of spinal ganglia that form C- fibres are further divided into two basic categories peptidergic and non-peptidergic (Hunt and Rossi, 1985, Snider and McMahon, 1998). Peptidergic neurons are named according to the expression of neuropeptides such as CGRP (peptide derived calcitonin gene) and SP (substance P). For the so-called

non-peptidergic cell is typical the binding site for the plant lectin IB4. Moreover, these cells express receptors for glial nerve growth factor (GDNF) and purinergic P2X3 receptors for ATP.
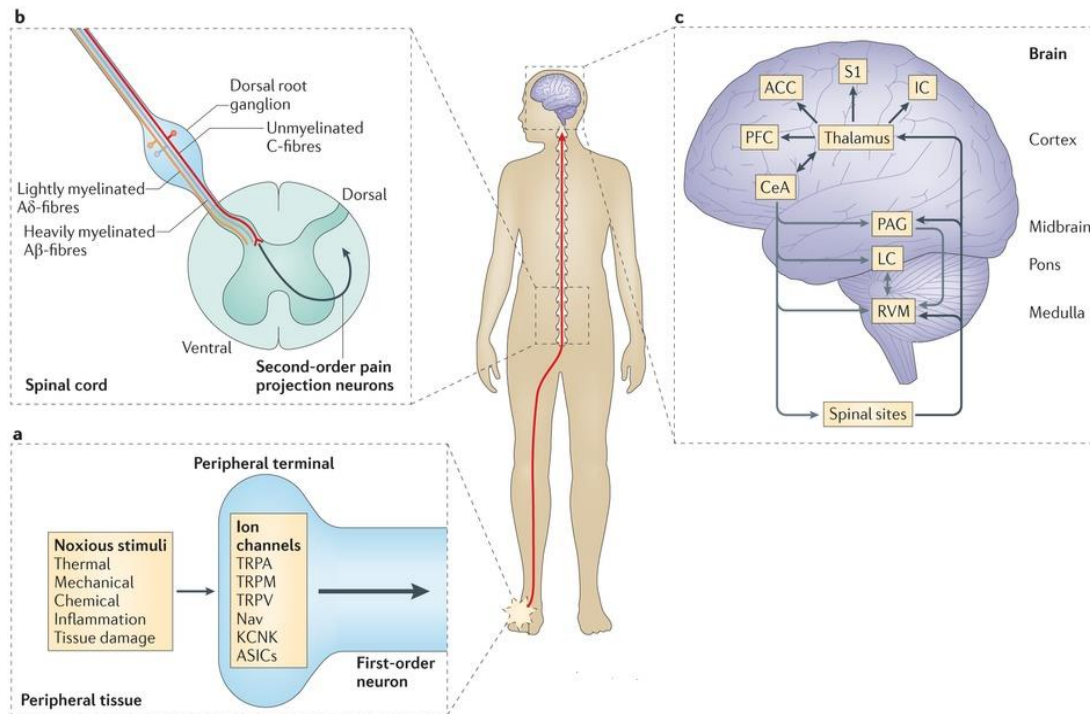
Figure 3. *a) Nociceptive signals are transmitted from the periphery by nociceptive sensory neurons (first-order primary afferent neurons) which peripheral terminals are express different ion channels. b) Action potentials are conducted along the axons of nociceptive fibres, through the cell body in the dorsal root ganglion to the axonal terminals, which form the presynaptic element of central synapses of the sensory pathway in the spinal dorsal horn or hindbrain. The central terminals of Aβ- and C- fibres form synapses with interneurons and second-order nociceptive projection neurons, primarily within the superficial laminae of the spinal dorsal horn. c) Third-order neurons in the thalamus project to several cortical and subcortical regions that encode sensory-discriminative - somatosensory cortex, emotional - anterior cingulate cortex, amygdala and insular cortex, and cognitive - pre-frontal cortex aspects of pain. Several brainstem sites also contribute to the descending modulation of pain - periaqueductal grey, locus coeruleus and rostral ventromedial medulla. Adopted from (Grace et al., 2014)*

Peripheral projections of peptidergic neurons innervate the deepest layer of the skin stratum spinosum, and their central projections are localized in lamina I and

the outer part of lamina II. Non-peptidergic neurons innervate the superficial layer of the skin stratum granulosum, and their central projections end preferably in the inner part of lamina II or III in the dorsal horn of spinal cord (Snider and McMahon, 1998, Zylka et al., 2005). The capability of nociceptors to detect different qualities of stimuli was made possible thanks to different receptors and ion channels on the cell surface and the specific set of expressed receptors that characterize individual populations of nociceptors.

In the dorsal horn of spinal cord are localized the first synaptic connections of the nociceptive pathway. For PAF, the glutamate is considered as the main neurotransmitter that binds to ionotropic and metabotropic glutamate receptors at postsynaptic neurons. Additionally neurons also synthesize a variety of molecules that can act as neuromodulators but their role is rather minor and modulating. These are neuropeptides SP and CGRP, ATP, NO diffusing gas; phospholipid metabolites Prostaglandins (PG); neurotrophins (such as NGF or GDNF) and other peptides (e.g. galanin, cholecystokinin (CCK) or neuropeptide Y) (Millan, 1999). First synapses allow pivotal modulation of the signal intensity coming from the periphery. This modulation may even cause extinction or amplification of signal and generate further action potentials on the secondary projection neurons.

2.2.2 Nociceptive processing at spinal cord level

The nociceptive information undergoes significant modulation at the spinal cord level in which both inhibitory and excitatory pathways play an important role. From the spinal cord level is nociceptive information further transmitted by projection – second order neurons to the brain.

The dorsal horn of the spinal cord is divided into six parallel layers (laminae) I - VI based on differences in the size and density of neurons (Rexed, 1952). **Lamina I** (marginal zone) engages most of the central terminals of Aδ fibers and contains most projection neurons. The bodies of projecting neurons are generally larger than the bodies of interneurons. **Lamina II** (substantia gelatinosa) is dominated by interneurons. Lamina II is subdivided in to an inner and an outer part IIi (inner) / IIo (outer). Laminae I and II are often referred to as the superficial layers of dorsal horn spinal cord, and they are the main projection areas of primary nociceptive neurons. Laminae III – IV are grouped under the name proprius nucleus. Some neurons in laminae V – VI, which represent the deep layers of spinal cord, are

also involved in nociceptive signalling. The anterior horn of the spinal cord is divided into laminae VII – IX that mostly feature neurons transmitting signal to motorneurons. Lamina X surrounds the central channel.

According to the response to the activity from PAF, projection neurons are divided into low threshold (LT), high threshold (HT) and a "wide dynamic range "(WDR) neurons. Low-threshold projection neurons respond preferentially, to non-noxious stimulation, such as light tactile stimuli conducted by Aβ primary fibers. High threshold neurons respond to painful stimuli transmitted by specific nociceptive neurons - C and Aδ fibers. They are concentrated in lamina I (IIo) and rarely occur in the deep layers (IV – VI). Neurons with wide dynamic range respond to signals ranging from painless to painful. They are excited by mechanical, thermal and chemical stimuli transmitted by C Aδ and Aβ fibers. Significant convergence of inputs occurs at these projection neurons. They are localized mainly in laminae IV-VI, and rarely in laminae I, IIo and X (Rexed and Strom, 1952, Millan, 1999).

Interneurons are traditionally divided into excitatory and inhibitory according to the principal neurotransmitters they release. The largest population of interneurons in the spinal cord is localized in lamina II. Excitatory interneurons allow primarily indirect polysynaptic activation of projection neurons. They release excitatory amino acids and a variety of neuropeptides. Inhibitory interneurons are in direct contact with projection neurons and primary afferent fibers, which allows the application of presynaptic and postsynaptic mechanisms of inhibition of nociceptive signalling coming from primary sensory neurons. They are distinguished based on the neurotransmitters they release – either GABA or glycine (Spike et al., 1993). Interneurons play a huge role in regulation of nociceptive signalling on spinal cord level.

2.2.3 Nociceptive processing in the brain

The highly complex ascending pain projection pathways can be divided into two types (Almeida and Lima, 1997, Millan, 1999). Monosynaptic pathways project directly in to cerebral structures (spinothalamic tract) whereas polysynaptic pathways possess a relayed to the third-order neurons (spinoreticular or postsynaptic dorsal column pathways). Somatosensory cortex is involved in the discriminatory aspect of pain and areas with association cortex projections are related to affective responses involving both somatic and autonomic motor systems(Millan, 1999). Many

supraspinal control areas – reticular formation, midbrain, thalamus, hypothalamus, amygdala, cingulate cortex, basal ganglia and cerebral cortex also modulate pain perception (Tracey and Mantyh, 2007). Neurons originating from these areas may activate neurons of the descending spinal pathways, which terminate in the DH. This network of cortical, subcortical, and brainstem structures also includes prefrontal cortex, anterior cingulate cortex, insula, periaqueductal grey, rostral ventromedial medulla, and dorsolateral pons/tegmentum (Tracey and Mantyh, 2007, Garland, 2012). The coordinated activity of these brain structures modulates nociceptive signals via descending projections to the spinal dorsal horn. The central nervous system can selectively control signal transmission from specific parts of the body (Garland, 2012).

The descending pain modulatory system has both anti- and pro-nociceptive effects. The descending pain modulatory system represents the means by which the central nervous system inhibits nociceptive signals at the spinal outputs (Heinricher et al., 2009). Was observed that direct electrical stimulation of the periaqueductal grey (PAG) could produce remarkable analgesic effects as evidenced by the ability to undergo major surgery without pain (Reynolds, 1969). Yet, this system can also facilitate nociception. Projections from the periaqueductal grey to the rostral ventromedial medulla have been shown to enhance spinal transmission of nociceptive information from peripheral nociceptors (Carlson et al., 2007).

2.2.4 Synaptic plasticity

In the mature nervous system, synaptic transmission can be modulated, potentiated or suppressed, in many ways. Synaptic strength describes the magnitude of post-synaptic currents or potentials in response to a pre-synaptic action potential and may be modified in an activity-dependent manner.

Synaptic plasticity is the ability of neural circuits to undergo changes in function or structural organization due to previous activity. It is considered to underlie both adaptive as well as maladaptive learning processes (Scholz and Woolf, 2002). It is important for normal events like memory formation and pathological processes such those underlying chronic pain and neurodegenerative disorders (Scholz and Woolf, 2002, Bredesen, 2009, Wang et al., 2009). A general rule of synaptic plasticity describes a basic mechanism of an increase in synaptic efficacy arises from the presynaptic neurons repeated and persistent stimulation of

the postsynaptic cell, and states. Echoing activity in transient assemblies of neurons carries a memory trace that becomes permanently laid down as changes in synaptic weights when a presynaptic neuron repeatedly or persistently takes part in firing towards the postsynaptic neurons (Bliss and Lomo, 1973). The rule implies both temporal and spatial constraints on storage of information manifested as synaptic plasticity (Froemke and Dan, 2002). It was demonstrated that the fundamental factors inducing synaptic plasticity involve temporal order of the synaptic input and the postsynaptic spikes within a narrow temporal window, back propagating action potential serving as a global signal for synaptic plasticity and a specific temporal patterns of postsynaptic activity burst (Paulsen and Sejnowski, 2000). Potentiation can occur even in the absence of the postsynaptic somatic spikes (Mehta, 2004).

2.2.5 Central sensitization

Prior to the discovery of central sensitization, the predominant view on pain processing in the central nervous system was of a mostly passive neural relay that conveys the information of the onset, duration, intensity, location and quality of peripheral noxious stimuli, encoded in action potentials - much like a telephone wire, from one site to another. Ongoing research showed that nociceptive peripheral terminals could be sensitized after injury by reducing their threshold, mainly to heat stimuli, and only at the site of injury, where the terminals were exposed to inflammatory modulators (Perl et al., 1976, LaMotte et al., 1982). This phenomenon is clearly a very important contributor to inflammatory pain hypersensitivity but it cannot account for dynamic tactile allodynia, the temporal summation of pain, or the secondary hyperalgesia. Some other explanation was needed. This turned out to be the increased synaptic function triggered within the CNS by nociceptive inputs (Woolf, 1991, Treede et al., 1992, Woolf and Salter, 2000).

Transformative breakthrough was the first description of long term potentiation (LTP) in the hippocampus (Bliss and Lomo, 1973). Brief high frequency coincident input produced a persistent increase in synaptic efficacy. Central sensitization represented a progressively increasing output during the course of a train of identical stimuli (Woolf, 2011). This way the input in one set of nociceptor sensory fibers amplified subsequent responses to other non-stimulated nociceptor or non-nociceptor fibers (Thompson et al., 1993).

The underlying neurobiological basic mechanism for central sensitization is that for most central circuits, the receptive field properties of neurons defined by the firing of action potentials is only the "tip of the iceberg". Most of the synaptic input to neurons is subthreshold (Woolf and King, 1989) Increasing synaptic strength by presynaptic increase in the excitatory transmitter release, by increase of post-synaptic response to the transmitter, by reducing inhibition or increasing membrane excitability can strengthen these normally subthreshold inputs to suprathreshold action potentials, producing profound changes in functional properties (Woolf and King, 1990, Woolf et al., 1994, Takazawa and MacDermott, 2010). More recently it has become realized that in addition to activity-dependent synaptic plasticity, changes in microglia, astrocytes, gap junctions, membrane excitability and gene transcription all can contribute to the maintenance of central sensitization (Chacur et al., 2009, Chang and Waxman, 2010, Chiang et al., 2010, Rivera-Arconada and Lopez-Garcia, 2010).

Central sensitization manifests as pain hypersensitivity, particularly dynamic tactile allodynia, secondary punctate or pressure hyperalgesia, aftersensations, and enhanced temporal summation.

2.3 Modulation of synaptic nociceptive transmission at spinal cord level

Chemically or physically activated peripheral nociceptive afferent fibres of DRG neurons transmit nociceptive signal to the dorsal horn of the spinal cord. After arrival of action potential, a diverse array of neurotransmitters, neuromodulators and other neuroactive substances can be released from the presynaptic central terminals, and bind simultaneously to different types of receptors on the postsynaptic membrane of spinal neurons and also on the presynaptic membrane itself (Millan, 1999, Willis and Coggeshall, 2004). These compounds can modulate neuronal excitability in a very complex manner.

Transmission may be affected by the amount of released transmitter or neuromodulator, by the density, types and kinetic characteristics of presynaptic and postsynaptic receptors or by, opening and closing of ion channels and activity of transporters. Each of these factors may be subject to a number of regulatory effects. Fast excitatory synaptic transmission is ensured by an outpouring of glutamate from the DH neurons, and binding to the postsynaptic ionotropic glutamate receptors. Action potential coming from the periphery can cause excitatory postsynaptic

currents (EPSC) that are mediated primarily by AMPA receptors. Postsynaptic NMDA receptors are also involved in rapid transmission, but they can be tonically inhibited by Mg^{2+} ions. The activation can occur only after removal of bound Mg^{2+} from the channel receptor, this depending upon depolarization of plasma membrane. Slow excitatory synaptic transmission is mediated by metabotropic receptors, activated either by glutamate or neuropeptides such as substance P and CGRP (Woolf and Salter, 2000).

2.3.1. Glutamate receptors

The main neurotransmitters at excitatory synapses are the excitatory amino acid (EAA) L-glutamate together with EAA L-aspartate. Glutamate acts as an agonist on a large family of glutamate receptors that can be divided in two basic groups on **ionotropic** and **metabotropic receptors**. Ionotropic glutamate receptors can be further divided based on their specific agonists into three groups of AMPA, kainate and NMDA receptors. Since modulation of excitatory postsynaptic transmission is mediated by AMPA receptors in the dorsal horn spinal cord, following chapters described in detail.

The AMPA receptors are important elements of synaptic plasticity involving AMPA receptors phosphorylation and trafficking. Functional properties of AMPA receptors, depends on their subunit composition, phosphorylation state, and the interaction with other membrane proteins (Hollmann and Heinemann, 1994). GluR subunits have consensus region for phosphorylation by calcium/calmodulin-dependent protein kinase II and PKC (CaMKII, PKC) (Barry and Ziff, 2002, Boehm and Malinow, 2005). Four subunit GluR1-GluR4 forms in various ratios heteromeric tetramers, different combinations of subunits represent different receptor subtypes. GluR2 subunit is crucial to the channel selectivity, if it is incorporated in the structure of the receptor, channel pass only monovalent cations. Without GluR2 are AMPA receptors permeable to sodium, potassium and to a much lower extent to calcium. It is known that presence of the GluR2 subunit, make it AMPA receptors impermeable for calcium and protecting neurons against excitotoxicity (Angulo et al., 1997, Kim et al., 2001) which implies its possibly significant role in synaptic plasticity. The AMPA receptors are the main charge carriers during excitatory postsynaptic potential (EPSP) generation.

At the spinal cord dorsal horn neurons the AMPA receptors are expressed in lamina I, IIi, IIo and activated by EAA binding and briefly depolarize the neuronal membrane (Antognini et al., 2002), which often leads to generation of an action potentials in second-order neurons. The activation of AMPA receptors is regulated by rapid onset of glutamate desensitization and together with action of excitatory amino acid transporters (EAATs) create neuronal protective mechanism guarding neurons against glutamate-induced excitotoxicity (Yamada and Tang, 1993, Matthews et al., 2003).

The NMDA receptor (N-methyl D-aspartate) is another important ionotropic glutamate receptor. The NMDAR consists of two NR1 and two NR2 subunits with four different isoforms (A-D) and a related gene family of NR3 A and B subunits have an inhibitory effect on receptor activity. Subunit and isoform composition influences function and permeability of receptor (Lakhan et al., 2013). NR1 and NR2 subunit types are needed for proper NMDA receptor function (Cull-Candy et al., 2001). The NMDA receptors are a cation channels, permeable to sodium, potassium and calcium. NMDA receptors also integrate neuronal circuitry and modulate synaptic plasticity. They have exceptionally high Ca^{2+} permeability and voltage-dependent sensitivity to Mg^{2+} block, which prevents NMDA receptor function at resting membrane potential. NMDA receptor-mediated Ca^{2+} fluxes occur only when Mg^{2+} block is removed by increasing AMPA receptor-mediated depolarization (around 0 mV) (Malenka and Bear, 2004). NMDA are requiring co-activation by two ligands, glutamate and glycine. This event leads to longer duration of the depolarization up to several hundred milliseconds (Lester et al., 1990). NMDA can act as a detector for membrane depolarization and synaptic transmission and can trigger synaptic plasticity (Rao and Sejnowski, 2001). The NMDA receptors are modulated by many endogenous and exogenous activators and play a key role in a wide range of physiological and pathophysiological processes including pain, where they are responsible for a pain amplification (Popik et al., 2000, Bespalov et al., 2001).

2.4 Transient receptor potential channels

The chemical, thermal or mechanical signals are transduced into action potentials by specialized receptors that are expressed in the free nerve endings.

Transient Receptor Potential (TRP) channels have been identified as one of the key molecular transducers of **thermal and painful stimuli**. In humans, twenty seven TRP channels are expressed in diverse types of cells and tissues. Among them, eleven were described as **temperature-sensitive**: vanilloid receptors TRPV1-TRPV4, melastatin receptors TRPM2, TRPM3, TRPM4, TRPM5 and TRPM8, TRPA1 and TRPC5 (Fernandez-Carvajal et al., 2011, Vriens et al., 2011, Saito and Tominaga, 2015). These so called "thermoTRPs" display distinct thermal thresholds. TRPV3 and TRPV4 are sensitive to moderate temperatures (25 – 35 °C) (Guler et al., 2002, Smith et al., 2002, Benham et al., 2003) while the **capsaicin receptor TRPV1** (Figure 4.) to noxious hot temperatures above 43 °C (Caterina et al., 1997, Tominaga et al., 1998). The thermal threshold for the activation of the TRPV2 channel, which is structurally similar to TRPV1, is even above 52 °C(Macpherson et al., 2007). Melastatin receptors TRPM2 – TRPM5 were described as heat sensors (Talavera et al., 2005, Togashi et al., 2006, Vriens et al., 2011) whereas TRPM8 and TRPC5 are known to be activated by cold. The temperature threshold for TRPC5 activation is between 37 and 25 °C (Zimmermann et al., 2011) while TRPM8 receptor is activated by cooling agents such as menthol and icilin and noxious cold temperatures below 25 °C (McKemy et al., 2002).

2.4.1 Physiological role of TRPV1

TRPV1 receptor (transient receptor potential vanilloid Type 1) was the first molecularly identified and cloned member of vanilloid subfamily of TRPV channels (Caterina et al., 1997). Vanilloid subfamily of TRPV channels consist of six members TRPV1-6 amongst which 4 were confirmed to be involved in nociceptive transmission - apart from TRPV1 also TRPV2, TRPV3 and TRPV4 receptor.

TRPV1 structure corresponds with the typical structure of TRP channels. It is a homotetramer (Figure 4) and each subunit has six transmembrane intersections (S1-S6) with a hydrophobic loop between α-helix S5 and S6, which is involved in formation of the central pore of the channel. In terms of the structure and the function the C- and N-terminus of the receptor are equally important. Both ends are

hydrophilic, aimed into the cytoplasm and constitute 70% of the receptors total mass (Moiseenkova-Bell et al., 2008).

N-terminus represents more than a half of one subunits size (in the rat it is formed by 432 amino acids). The N-terminus contains a proline-rich sequence and six ankyrin domains (Caterina et al., 1997). One ankyrin domain consists of 33 amino acids organized in two antiparallel α-helices, separated by a loop (Figure 5). In TRPV1 receptor, ankyrin domains are involved in ATP and Ca^{2+} / calmodulin binding (Lishko et al., 2007).
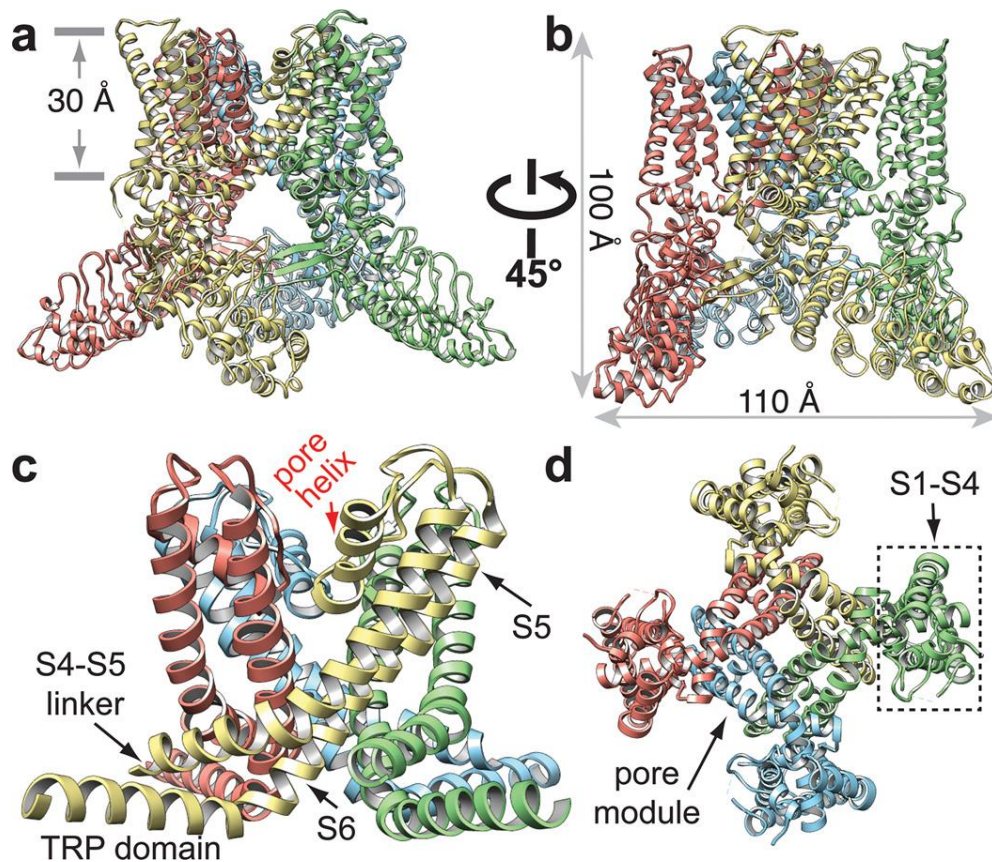
Figure 4. TRPV1 symmetric architecture. Ribbon diagram of TRPV1 atomic model with each of the four identical subunits color-coded, showing views from side (a and b). Ribbon diagram focusing in on side (c) view of S5-P-S6 pore with TRP domains. Bottom view focusing on transmembrane core (d), including S1–S4, S5-P-S6, and TRP domains. Adapted from (Liao et al., 2013).

C-terminus is very important for sensitization and desensitization of receptor (Figure 5), because it contains a calmodulin binding site, PIP2 and ATP binding domains and amino acid residues for phosphorylation (Touska et al., 2011). Deletion

of the distal part of the C-terminus (31 or 42 amino acids) had no significant effect on the sensitivity to a specific receptor agonist capsaicin or low pH, but strongly affected TRPV1 receptor sensitivity to thermal stimuli. Such deletions reduced the threshold by 3-9 °C. Whole C-terminus deletion leads to complete loss of sensitivity to chemical, thermal and voltage stimulus (Vlachova et al., 2003). In the proximal part of the C-terminal segment is located the domain responsible for tetramerization of TRPV1 subunits, and for this segment is characteristic the presence of TRP box with a highly conserved amino acid sequence (Garcia-Sanz et al., 2004).
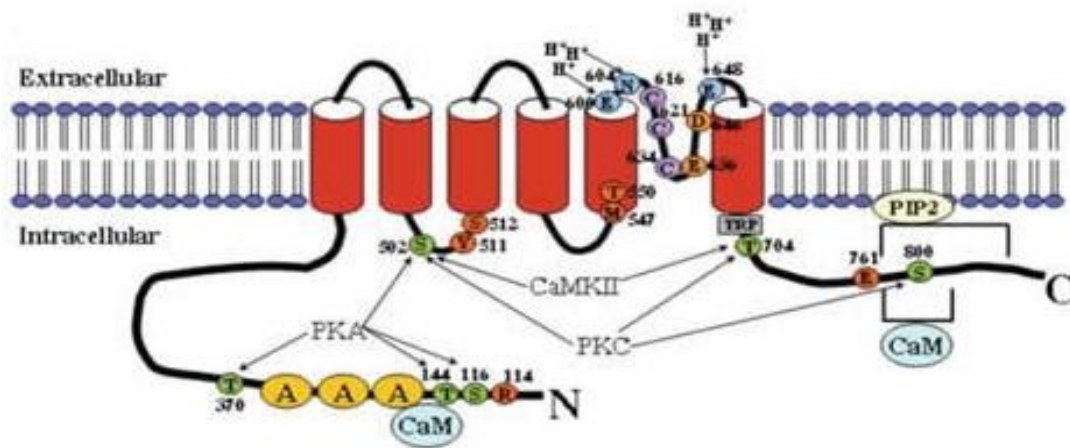
Figure 5. *A schematic diagram of a TRPV1 subunit in a bilayer. The subunit has six transmembrane domains (red) and a pore loop. The functional TRPV1 receptor is believed to form a tetramer. Residues involved in vanilloid binding (orange). "A" indicates ankyrin repetitions (yellow). Residues susceptible of phosphorylation (green). Two calmodulin-binding regions in the N- and C-termini are indicated by "CaM." Blue residues in the pore loop represent proton a table amino acids. Cysteine residues in the P-loop are susceptible of reduction (purple). PIP2 is shown to bind to the region indicated in the C-terminus. The TRP box represents the TRP domain. Adopted an adjusted from (Tominaga and Tominaga, 2005).*

TRPV1 has been widely studied in connection with pain mechanisms. It is located on subsets of nociceptors and is activated by numerous stimuli that are perceived as painful or noxious, including capsaicin, the active ingredient in hot chili peppers, as well as by protons, heat > 42 °C, and a family of endogenous-inflammation-associated lipids (Figure 6) (Nagy et al., 2014). The sensation evoked in humans by activation of TRPV1 in skin by capsaicin is burning (Simone et al., 1989); whereas, when applied into muscle, it is perceived as deep aching or cramping

pain (Marchettini et al., 1996) matching patient complaints associated with paclitaxel treatment (Boyette-Davis and Dougherty, 2011, Boyette-Davis et al., 2013). Consistently with these observations, TRPV1 expression is increased in rat dorsal root ganglia (DRG) after paclitaxel treatment when thermal hyperalgesia is observed and the use of a TRPV1 antagonist was shown to reduce this hypersensitivity (Hara et al., 2013). Ligands of guanine nucleotide binding protein-coupled receptors (GPCR) that activate phospholipase C (PLC) leading to hydrolysis of phosphatidylinositol 4,5, bisphosphate (PIP2) disinhibit the TRPV1 channels (Huang et al., 2002). TRPV1 sensitization resulting in neuropathic pain depends on channel phosphorylation by protein kinase C (PKC) or protein kinase A (PKA) (Rathee et al., 2002) and this process may be triggered by products of inflammation, including prostaglandin E2 (PgE2), bradykinin, adenosine triphosphate (ATP), and serotonin (5-HT). Nerve growth factor (NGF) acting via tyrosine kinase A (trkA) receptors increases TRPV1 expression (Cesare et al., 1999, Premkumar and Ahern, 2000, Vellani et al., 2001).

TRPV1 receptor is expressed in different amount in the entire organism. In humans, the TRPV1 receptor was detected in DRG, brain, kidney, pancreas, testis, uterus, spleen, stomach, small intestine, lung, liver and numerous of others organs. Surprisingly it was found that expression of TRPV1 mRNA in brain and peripheral tissues is much lower than in the DRG (Hayes et al., 2000). TRPV1 positive central endings in the spinal cord are mainly located in laminae I and II, while the inner lamina IIi contains larger quantities than lamina IIo (Guo et al., 1999, Valtschanoff et al., 2001).Distribution of TRPV1 receptors is not constant over the life time. Changes of TRPV1 expression in DRG neurons were documented during ontogeny and adolescence (Cavanaugh et al., 2011b). After numerous studies showed that the TRPV1 is also expressed in the brain and the spinal cord (Jancso-Gabor et al., 1970, Szallasi and Blumberg, 1991, Szallasi et al., 1994, Szallasi et al., 1995), scientists began investigating their role in the CNS. The main question was, how are the TRPV1 receptors activated in these areas? Under normal circumstances, there are no dramatic changes in inner environment of the CNS (such as changes in temperature, pH, presence of high concentrations of alkaloids, injury etc.) that are considered to activate TRPV1 receptors in periphery. Neurotransmitter anandamide was the first documented endogenous activator of TRPV1 receptors (Zygmunt et al., 1999).
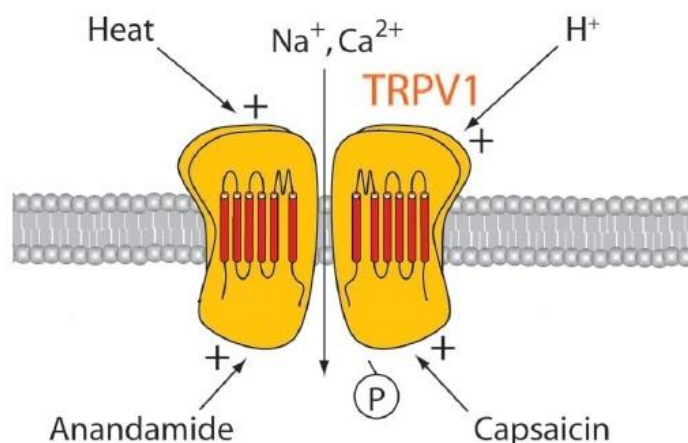
Figure 6. TRPV1 is activated by noxious heat (up to 43 °C), low pH (<5.9), eicosanoids, anandamide, and capsaicin and phosphorylated by protein kinases. Adapted and adjusted from (Benarroch, 2008).

Furthermore, it was found that some eicosanoids and leukotrienes may function as endogenous activators of TRPV1 – such as lipidic substances derived from arachidonic acid (AA). Also some products of lipoxygenases can block effect of the antagonist capsazepine, which suggests that they compete for the same binding site with capsaicin (Hwang et al., 2000). Another endogenous activator is the 20-hydroxyeicosatetraenoic acid (20-HETE), which is also a derivate of arachidonic acid. The 20-HETE activates the TRPV1 receptor through a binding site other than the one for capsaicin. Activating effects of 20-HETE can be reduced by administration of selective inhibitor of protein kinase A (PKA) and C (PKC). TRPV1 can be also endogenously activated by linoleic acid metabolites that can contribute to the formation of inflammatory hyperalgesia (Patwardhan et al., 2009). Other endogenous agonists of the TRPV1 receptors are N-arachidonoyl-dopamine (NADA) and N-oleoyl-dopamine (OLDA). Both these substances induce massive influx of Ca^{2+} into the cell and their effect can be blocked by capsazepine. Their intradermal administration results in decrease of thermal threshold and development of thermal hyperalgesia in rats (Huang et al., 2002, Chu et al., 2003). OLDA can significantly influence synaptic transmission in the DH spinal cord (Spicarova and Palecek, 2009). Results suggest that the activation of TRPV1 receptors by low concentrations of endogenous agonists, such OLDA, can have a significant effect on the modulation of synaptic transmission in the DH especially during pathophysiological conditions when TRPV1 are sensitized.

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2.4.2. TRPV1 receptor in nociceptive transmission

TRPV1 receptors are well recognized as molecular integrators of nociceptive stimuli at peripheral nerve endings (Matta and Ahern, 2007) . The vast majority of spinal TRPV1 receptors are located on central branches of small and medium size DRG neurons in the superficial dorsal horn of spinal cord (Guo et al., 1999, Cavanaugh et al., 2011b). Spinal TRPV1 receptors were shown to have an important role in modulation of nociceptive transmission, especially under pathological conditions (Lappin et al., 2006, Spicarova and Palecek, 2008, Chen et al., 2009, Spicarova and Palecek, 2009). It was suggested that they become phosphorylated and increase their sensitivity to endogenous agonists (Spicarova and Palecek, 2008, 2009, 2010).

TRPV1 receptors have been also identified as the molecular integrator of inflammatory pain (Cortright and Szallasi, 2004). TRPV1 receptors are located on peripheral and also central terminals of primary nociceptive neurons. Peripheral TRPV1 receptors are activated by thermal stimuli and protons, while many other substances can sensitize TRPV1 receptors and moderate their expression. Inflammatory mediators, such as bradykinin, can reduce the threshold for TRPV1 activation to the temperature within physiological values (Sugiura et al., 2002). NGF also affects the sensitivity of TRPV1 receptors, which is supported by the fact that the thermal hyperalgesia induced by NGF does not develop in TRPV1$^{-/-}$ knock out mice. TRPV1 receptors have a pivotal role in inflammation-induced thermal hyperalgesia (Caterina et al., 2000). The number of TRPV1 receptors may be increased by application of NGF during inflammation (Ji et al., 2002). After experimentally induced inflammation it was also observed that the number of unmyelinated peripheral fibers immunopositive labelled for TRPV1 receptors increase (Carlton and Coggeshall, 2001). This is consistent with increased axonal transport of mRNA encoding TRPV1 receptors associated with increased sensitivity of primary afferents to capsaicin and the finding that activated PKC causes rapid insertion of TRPV1 receptors to the plasma membrane (Tohda et al., 2001, Morenilla-Palao et al., 2004).

Thermal hyperalgesia induced by nerve growth factor nerve (NGF) is at least partially caused by the phospholipase C and protein kinase C dependent phosphorylation of TRPV1 receptors. Activation of PLC leads to hydrolysis of

membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) (Huang et al., 2002). Sensitization of TRPV1 receptors by activation of PKA may also have an important role in inflammation induced hyperalgesia. PKA takes part in a signal cascade leading to activation of metabotropic receptors for prostaglandins (Rathee et al., 2002). There are also other mediators of inflammation that can activate TRPV1 receptors through an indirect route via products of lipoxygenase like bradykinin. Bradikinin binds to B2 receptor and excites nociceptors via the production of arachidonic acid metabolites (12-HPETE), which then activate TRPV1 receptors (Hwang et al., 2000).

TRPV1 receptor channels are permeable to monovalent cations (Na^+) as well as for Ca^{2+}. Their influx into the cytosol activates protein kinase dependent on Ca^{2+} and calmodulin (CaMKII), which, like the PKA and PKC phosphorylation, sensitizes TRPV1 receptors. The influx of Ca^{2+} ions also contributes to the change of distribution of positive and negative charges on the inner and outer side of the plasma membrane, which leads to its depolarization and may subsequently induce formation of action potential. TRPV1 receptors significantly contribute to the sensitization of free nerve endings and thus greatly facilitate the transmission of nociceptive signals to the CNS. The desensitization phase that occurs after TRPV1 receptors activation is also important for pain transmission. This loss of sensitivity to activators is mostly dependent on the intracellular concentration of Ca^{2+} ions (Koplas et al., 1997). A significant role in the process of desensitization of TRPV1 receptors plays the regulation of calcineurin (Docherty et al., 1996). Activation of TRPV1 receptors on peripheral nociceptors by capsaicin in high concentration leads to desensitization and possible degeneration of sensory endings, and can induce prolonged local anesthesia (Simone et al., 1998, Uchytilova et al., 2014).

2.4.3 Mechanisms of modulating TRPV1 receptor

TRPV1 receptors are often called molecular integrators of nociceptive stimuli. They not only directly detect the nociceptive stimuli, but also have big influence on activation through second messengers. Among the modulators acting indirectly included e.g. ATP, bradykinin, glutamate, histamine, trypsin, serotonin, nerve growth factor (NGF), prostaglandins, tumor necrosis factor (TNF), glial nerve growth factor (GDNF), insulin and insulin derived from growth factor 1 IGF-1 (Gunthorpe and Chizh, 2009, Premkumar and Abooj, 2013). These substances

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activate TRPV1 receptors and trigger the signal cascade that leads to the activation of protein kinases that in their turn phosphorylate the receptor. Thus cause the sensitization e.g. protein kinase C (PKC), the cAMP-dependent protein kinase A (PKA), Ca^{2+} / calmodulin-dependent kinase II (CaMKII), mitogen-activated protein kinase (MAPK) or phosphatidylinositol-3 kinase (PI_3K).

Equally important role have also those modulators which are directly connected with TRPV1 receptors. These include primarily intracellular ATP, calmodulin, and phosphatidylinositol-4,5-bisphosphate (PIP2). Many inflammatory mediators play an important role in the hyperalgesia as well. One of them is **Bradykinin**. The main effect of bradykinin is the modulation of TRPV1 receptor through PLC and PLA2 (Ferreira et al., 2004). PKC phosphorylates the TRPV1 and causes the sensitization of nociceptors by decreasing the temperature threshold required for TRPV1 receptor activation within physiological values (Cesare and McNaughton, 1996, Sugiura et al., 2002), whereas PLA2 cleaves membrane phospholipids, produces AA and this process results in endogenous activation of TRPV1 receptor (Thayer et al., 1988). Bradykinin is capable not only to increase the sensitivity of TRPV1 receptors for thermal stimuli (Sugiura et al., 2002), but also to increase their response to capsaicin in DRG neurons (Vellani et al., 2001).

Another modulator of TRPV1 is **adenosine triphosphate (ATP),** which can influence the receptor in several ways – the extracellular ATP through metabotropic P2Y1 receptors and the intracellular ATP by direct interaction with TRPV1 (Tominaga et al., 2001, Lishko et al., 2007). In rat DRG cells was observed that the extracellular ATP can trigger a cascade of second messengers via P2Y1 receptor leading to the activation of PKC. Consequently, it phosphorylates the TRPV1 receptor and reduces the threshold for activation from ~43 °C to only 35 °C (Tominaga et al., 2001). In contrast, the intracellular ATP induces sensitization through direct binding to the TRPV1 receptor. A binding site was found in the first to third ankyrin domains at the N-terminus. This binding site can be occupied not only by ATP but also by calmodulin (CaM) in complex with Ca^{2+}. These two substances compete for the same binding site and have the opposite effect on the receptor. While ATP induces sensitization, Ca^{2+}/CaM have more inhibitory effects (Lishko et al., 2007). Eicosanoids, leukotrienes and **prostaglandins (PGs)** are lipid substances produced by cyclooxygenase from AA. PG interacts with TRPV1 through metabotropic receptors coupled with trimeric G proteins that modulates its

properties. Prostaglandin E2 (PGE2) and prostaglandin I2 (PGI2) have the greatest influence on the processing of painful sensations and development of hyperalgesia via the PGE2 - EP1-4 receptor and the PGI2 - IP receptor. The EP1 receptor stimulates PLC which causes the mobilization of intracellular stores of Ca^{2+} and enhances PKC activity. EP2 and EP4 activate the adenylate cyclase (AC), which produces cyclic adenosine monophosphate (cAMP) and activates PKA. The IP receptor activates AC as well as PLC (Narumiya et al., 1999, Kawabata, 2011). The sensitization of TRPV1 receptors can be also induced by the nerve growth factor (NGF) (Shu and Mendell, 1999). NGF acts via the TrkA receptor with tyrosine kinase activity. After the activation by NGF, a complicated signalling pathway is trigger. The activation of PI3K and MAPK has proven to be particularly crucial for sensitization of TRPV1 receptor to capsaicin after acute administration of NGF. TrkA receptor activation leads to the activation of PLC and PKC (Bonnington and McNaughton, 2003, Zhu and Oxford, 2007). An important modulator of TRPV1 receptor is also membrane phospholipid **phosphatidylinositol-4,5-bisphosphate (PIP2),** and his secondary messengers IP3 and DAG. PIP2 binding site is located in the proximal part of the C-terminus and in the intracellular loop between transmembrane subunits S4 - S5. Effect of PIP2 on the TRPV1 receptor is still considered to be little controversial.

Low pH has also a significant modulatory effect on TRPV1 receptor. H^+ themselves have the ability to activate TRPV1 receptor, but decreased pH (from pH 7.6 to pH 6.3) markedly enhances the receptors response to capsaicin (Caterina et al., 1997). Reduced pH also affects the activation threshold for thermal stimuli, for example at pH $\leq$ 5,9 TRPV1 receptors are activated already at room temperature (22 °C) (Tominaga et al., 2001). From these observations it follows that milder acidosis, occurring during ischemia and inflammation, sensitizes TRPV1 receptor while significant decrease of pH can directly activate the receptors.

The function of TRPV1 receptors in central endings of primary nociceptive neurons may be also modulated by **glutamate** alone. Glutamate is released from central presynaptic endings of DRG neurons in the spinal cord and can also activate presynaptically localized metabotropic mGluR5 receptors. These can subsequently activate PLC and cause enhanced production of DAG and IP3. The activation of mGluR5 receptors results in increased frequency of mEPSC in spinal neurons. This increase was completely inhibited when TRPV1 were blocked (Kim et al., 2009b).

2.5 PAR2 in modulation of nociceptive signalling at the spinal cord level

Extracellular proteases such as thrombin, trypsin, serin protease 1 and coagulation factors VII and Xa can regulate target cells by cleaving and activating receptors that belong to a family of G-protein-coupled protease-activated receptors (PARs) (Brass and Molino, 1997, Corvera et al., 1997, Molino et al., 1997, Camerer et al., 2000, Vergnolle, 2000). This receptor family has at least four members (PAR1–4). Mechanism of receptor triggering involves proteolytic unmasking of a cryptic N-terminal sequence on the extracellular membrane that acts as a tethered receptor-activating ligand (Figure 7). In general, several proteases can activate a single PAR by cleaving it at specific site and exposing the tethered ligand domain. For example, trypsin, tryptase, coagulation factors VIIa and Xa and certain membrane-anchored proteases can all cleave and activate PAR2, although with varying potencies (Cenac et al., 2003, Chen et al., 2011, Rothmeier and Ruf, 2012).

There are short specific synthetic peptides, based on the tethered ligand sequences, designed as PARs 1,2 and 4 agonists (Brass and Molino, 1997). Some authors consider PAR3 as a co-factor for the activation of other PARs and it has no selective activating peptide (Noorbakhsh et al., 2003, Zhao et al., 2014). These synthetic peptides have been shown to activate the specific receptors and mimic the effects of the activating proteases for example for PAR2 are frequently used SLIGKV-NH2 or 2-Furoyl-LIGRLO-amide. Due to these selective peptides, the physiological consequences of activating different PARs may be studied and distinguished accurately (Hollenberg et al., 1997, Kawabata et al., 1999). For the purpose of research was also developed a compounds with are able acting as antagonists of PAR2 e.g. FSLLRY-NH2 or GB83.

PARs are present in most body tissues, with the highest expression in the epithelium (lungs, liver, digestive tract, skin, blood vessels) and they are also present in the peripheral and central nervous system (Dery et al., 1998, Vergnolle et al., 2001b, Hollenberg and Compton, 2002). The physiological effect of their activation varies in different tissues.
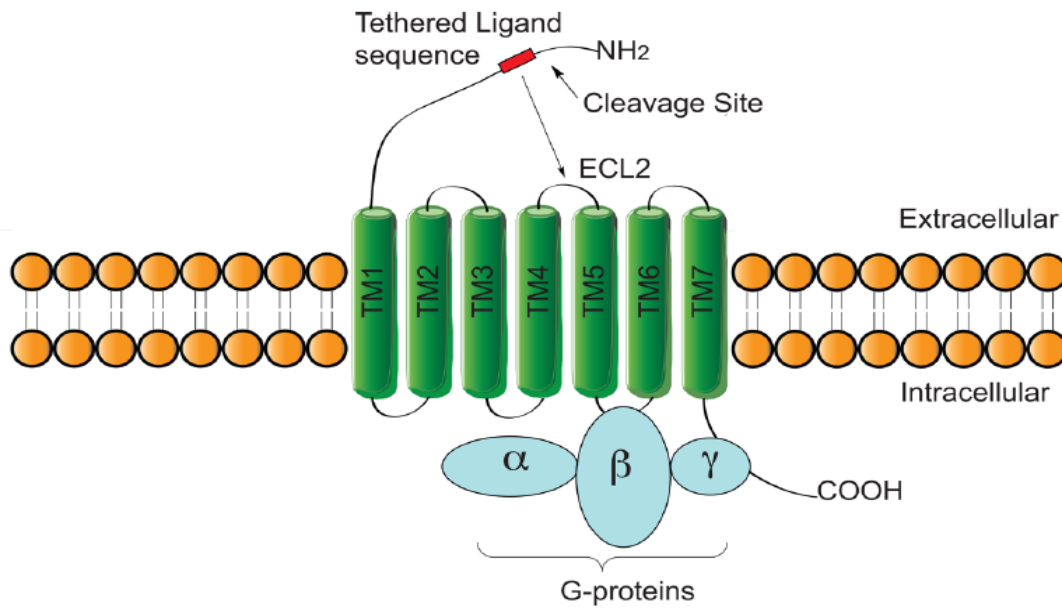
Figure 7. *Activation of PAR2. The tethered ligand (red), exposed through cleavage by proteases, folds back onto extracellular loop 2 (ECL2) and/or a specific 7TM region to activate the receptor. Adapted from (Yau et al., 2013)*

PAR2 receptors are known to play important roles in the organism response to tissue injury, notably in the process of inflammation and repair (Dery et al., 1998). In particular, agonists of PAR2, tryptase and trypsin released from different cell types including mast cells, have widespread proinflammatory effects in part via a neurogenic mechanism (Saifeddine et al., 1996, Vergnolle et al., 1999, Steinhoff et al., 2000, Seeliger et al., 2003). PAR2 are expressed on a subset of primary sensory neurons and PAR2 agonists stimulate release of substance P (SP) and calcitonin gene-related peptide (CGRP) in peripheral tissues via activation of peripheral nerve endings (Steinhoff et al., 2000). It was also reported that PAR2 activation can sensitize adult rat dorsal root ganglion (DRG) neurons *in vitro* (Steinhoff et al., 2000). Intraplantar injection of subinflammatory doses of PAR2 agonists in rat and mice induced thermal and mechanical hyperalgesia and elevated Fos protein expression in the spinal cord (Vergnolle et al., 2001a). All these changes indicate an important role for PAR2 in nociceptive transmission.

2.5.1 Mechanisms of PAR2 activation and intracellular signal transduction

PARs are G-protein-coupled receptors (GPCRs), a family of receptors with large seven-transmembrane helical domain protein that sense molecules outside the

cell and activate intracellular signalling pathways (Nystedt et al., 1994, Hoogerwerf et al., 2001, Macfarlane et al., 2001). Thrombin and trypsin are usually regarded as the main activators of PARs. PAR2 is a target for trypsin and other serine proteases, such as mast-cell tryptase, but is not activated by thrombin (Nystedt et al., 1995, Nystedt et al., 1996, Molino et al., 1997). PAR2 lacks a hirudin-like thrombin-binding domain, but trypsin cleavage site was detected in its extracellular N-terminus domain (Nystedt et al., 1995). PAR2 is activated by proteolytic cleavage of its extracellular amino terminus (Hollenberg et al., 1997, Steinhoff et al., 2000). These receptors can be also activated by exogenous proteases, as well as non-proteolytically by exogenous peptide sequences that mimic the final amino acids of the tethered ligand (Hollenberg et al., 1997, Al-Ani et al., 1999).

The signalling pathways that are triggered by PAR2 activation through the binding to activation molecules on G-proteins (Gq/11, Gi/o) may activate several signalling pathways including phospholipases, Jun N-terminal kinase and p44/42 mitogen-activated protein kinase (MAPK), ERK1/2, phosphatidylinositol 3-kinase (PI3K), adenylate cyclase (AC), protein kinase C (PKC), protein kinase A (PKA) and members of the SRC-family of tyrosine kinases (Kanke et al., 2001, Suen et al., 2010, Chen et al., 2011, Suen et al., 2014, Bao et al., 2015). These signaling pathways can affect various cellular activities – proliferation, gene transcription, morphological changes, motility and survival (Macfarlane et al., 2001, Kawabata et al., 2004). PAR2 activation was also reported to result in up/down-regulation of about 2500 genes that are important mainly for cell metabolism (around 1000), cell cycle, complement and MAPK pathway, sirtuin enzymes, histone deacetylases and inflammatory cytokines (Suen et al., 2010).

2.5.2 Expression of PAR2 in the nervous system

High density expression of PAR2 receptors was documented in neurons of hippocampus, cortex, amygdala, thalamus, hypothalamus, striatum and in DRG neurons in rats (Striggow et al., 2001). All four PARs are also present in astrocytes culture from rat brain (Wang et al., 2002a). PAR2 are expressed on neurons and astrocytes also in human CNS (D'Andrea et al., 1998, Noorbakhsh et al., 2006). Localization on guinea-pig myenteric and submucosal neurons was demonstrated earlier (Corvera et al., 1999). For the presence of PAR2 in the spinal cord dorsal horn exist mainly functional electrophysiological evidence (Alier et al., 2008, Fujita et al.,

2009, Huang et al., 2011), while recently PAR2 were detected also using western blot analysis in the rat spinal cord tissue (Chen et al., 2015). PAR2 were immunohistochemically detected in many small-sized and some medium- to large-sized DRG neurons (Steinhoff et al., 2000, Dai et al., 2004). Significant population of small DRG neurons expressing TRPV1 receptors, also showed expression of PAR2 (Amadesi et al., 2004, Dai et al., 2004), suggesting possible interactions between them.

2.5.3 Activation of PAR2 in neuronal tissue

Trypsin, tryptase are known as the main PAR2-activating proteases. Potentially, the main source of tryptase to activate neural PAR2 are mast cells that have been found in the choroid plexus, in the parenchymal and perivascular areas in the CNS and in close contact with peripheral nerves (Stead et al., 1987). Mast cell tryptase, although being less potent than trypsin, can regulate neuronal activity by cleaving PAR2 (Steinhoff et al., 2000, Reed et al., 2003) and other receptors. Selective PAR2 antagonist (FSLLRY-amide) was able to block paclitaxel induced neuropathic pain that was accompanied by mast cell tryptase activity in spinal cord and DRG in mice (Chen et al., 2011). Tryptase is thus a strong candidate for neuronal PAR2 activation. There is a number of other possible PAR2 activators, such as P22, another trypsin-like serine protease with PAR2-activating capacity that has been detected in rat brain (Sawada et al., 2000). Precursors of trypsin (trypsinogen-IV, trypsinogen-III) and other PAR2 activators (factor X) are also expressed in brain and human neural cell lines (Wiegand et al., 1993, Shikamoto and Morita, 1999).

2.5.4 Function of PAR2 in the nervous system

All four PARs are expressed throughout the peripheral and central nervous system and were suggested to play many different roles in neurogenic inflammation, pain perception, pruritus sensation, nerve regeneration, secretory functions and Ca^{2+} mobilization (Corvera et al., 1999, de Garavilla et al., 2001, Linden et al., 2001, Noorbakhsh et al., 2003). The role of PAR2 on the peripheral terminals of nociceptive DRG neurons is relatively well known. They have been implicated in the activation and/or modulation of nociceptor function (Vergnolle et al., 2001a, Fiorucci and Distrutti, 2002, Cenac and Vergnolle, 2005). The peripheral nerve endings may be activated by proteases which are generated and released during tissue trauma and

inflammation. Increased PAR2 immunoreactivity has been reported on primary afferent nerve fibers in the skin in patients with atopic dermatitis. Intracutaneous injection of a PAR2 agonist provoked an itch response in these patients (Steinhoff et al., 2003).

PAR2 agonists induced and prolonged hyperexcitability of guinea-pig submucosal neurons (Reed et al., 2003). PAR2 activation rapidly increased cytosolic concentration of Ca^{2+} from intracellular stores, whereas sustained Ca^{2+} elevation was dependent on the influx from extracellular space in adult rat DRG neurons *in vitro* (Steinhoff et al., 2000). Over 60% of DRG neurons coexpressed PAR2 with SP and CGRP, and PAR2 agonists stimulated the release of these peptides in peripheral tissues and in the spinal cord (Steinhoff et al., 2000). The release of these neuropeptides is important for nociceptive transmission. Intraplantar administration of subinflammatory doses of PAR2 agonists enhanced and prolonged hyperalgesia and induced activation of second-order nociceptive neurons at a spinal cord level in rodents. Also experiments in PAR2-deficient mice proved that PAR2 activation significantly contributes to inflammatory hyperalgesia (Vergnolle et al., 2001a).

Activation of PAR2 receptors triggers several intracellular signalling cascades, one of them is associated with G-protein and PLC/Ca^{2+}/PKC signalling pathway (Bohm et al., 1996, Seatter et al., 2004) and another one is associated with β-arrestin including MAPK (ERK1/2) signalling (DeFea et al., 2000, Rothmeier and Ruf, 2012). Activation of PKC may lead to the activation and nuclear translocation of NF-κB in neurons (Lilienbaum and Israel, 2003). Activation of NF-κB - mediated signalling increased the histone acetylation and facilitated the expression of BDNF in the central neurons (Peng et al., 2011). Some groups observed that activation of PAR2 signalling was required for the inflammation induced BDNF release from microglia (Yuan et al., 2010, Fan et al., 2014).

2.5.5 The role of PAR2 at the spinal cord level

Although PAR2 are known to be expressed on neurons and astrocytes in rodent and human CNS (Noorbakhsh et al., 2006), their expression in the spinal cord was not proved by immunohistochemical methods (Alier et al., 2008). However, functional studies on spinal cord slices suggested PAR2 presence on the central terminals of primary afferent nerve fibers and in dorsal horn neurons (Fujita et al.,

2009, Huang et al., 2011). Very recently PAR2 were detected by western blot analysis in the superficial dorsal horn tissue (Chen et al., 2015).

PAR2 are co-localized with proinflammatory neuropeptides, such as SP and CGRP, in DRG sensory neurons. Trypsin, tryptase and selective agonists of PAR2 stimulate the release of both CGRP and SP from C-fibers in peripheral tissues and in the spinal cord (Steinhoff et al., 2000). Peripheral administration of trypsin, tryptase and PAR2-activating peptide caused SP and CGRP release from sensory nerves. It caused massive edema that could be prevented by NK1R and CGRP1 receptor inhibition (Steinhoff et al., 2000).

Further studies at spinal cord level demonstrated that intrathecal (i.t.) application of PAR2 agonist induced mechanical allodynia and thermal hyperalgesia in healthy animals and augmented increased sensitivity present in a peripheral inflammatory pain model (Alier et al., 2008, Huang et al., 2011). PAR2-induced hypersensitivity was mediated by PGE2 release via COX activation in the spinal cord (Koetzner et al., 2004). In a paclitaxel-induced neuropathic pain model, i.t. application of PAR2 antagonist reversed mechanical allodynia and heat hyperalgesia (Chen et al., 2011).

Electrophysiological study with whole-cell recordings from young rat substantia gelatinosa neurons, showed no increase in sEPSC frequency or neuronal excitability following application of synthetic PAR2 agonists or trypsin. Moreover, trypsin slightly decreased sEPSC frequency in the dorsal horn (Alier et al., 2008). In comparison, another study showed that PAR2 agonist significantly enhanced frequency of sEPSC in a similar preparations from adult rats, suggesting involvement of PAR2 receptors in modulation of nociceptive synaptic transmission at the spinal cord level (Fujita et al., 2009). Direct activation of PAR2 in the spinal cord was suggested also in a study demonstrating decrease of spontaneous inhibitory postsynaptic currents frequency and amplitude in substantia gelatinosa neurons, implicating that PAR2 activation in the spinal cord may potentiate nociception by affecting the inhibitory rather than the excitatory transmission in the spinal dorsal horn neurons (Huang et al., 2011).

Altogether, this published evidence suggests that PAR2 play an important role in the process of nociceptive transmission and in neurogenic inflammatory mechanisms both in the periphery and at the spinal cord level. The precise role and

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exact importance for spinal nociceptive modulation is not completely understood and needs further research.

2.5.6 PAR2-induced activation of downstream protein kinases and other enzymes in neural tissue

PAR2 are expressed in nociceptive DRG neurons (Dai et al., 2004) and co-localizes with PKC and PKA, which are activated downstream of PAR2 receptors (Amadesi et al., 2006). The PKC activation cascade involves PAR2-induced activation of PLC leading to production of diacylglycerol and inositol 1,4,5-trisphosphate (Figure 8) which increases intracellular concentration of Ca^{2+} and activates PKC (Mule et al., 2002). The PKA activation cascade involves PAR2-induced mobilization of cAMP (Amadesi et al., 2006).

A selective PAR2 agonist can induce phosphorylation and activation of protein kinase D (PKD) in cultured rat DRG neurons (Amadesi et al., 2009). DRG neurons express all three isoforms of PKD, PKD1 (also known as PKCμ), PKD2 and PKD3. PKDs are downstream targets of diacylglycerol (DAG) and PKCs, which may be induced by PAR2 activation (Amadesi et al., 2009). PKD1 directly interacts with TRPV1 in cell lines and primary sensory neurons, suggesting that PKD may regulate TRPV1 activity (Wang et al., 2004) .

PAR2 activation leads to increased PLC activity and generation of IP3 and mobilization of Ca^{2+} in neurons and astrocytes (Rothmeier and Ruf, 2012), which can result in PLA2 activation and increased production and release of arachidonic acid (AA), which is the substrate for COX (Poole et al., 2013). An increase in AA production leads also to increased synthesis and release of PGE that through stimulation of AC may increase intracellular cAMP. Subsequently these steps lead to neuronal hyperexcitability and hyperalgesia (Koetzner et al., 2004).

PAR2 agonists lead to activation of ERK1/2 MAP kinases in multiple ways. It is known that the ERK/MAPK activation can contribute to increased nociceptive responses in the dorsal horn and DRG neurons following inflammation or nerve injury (Ji et al., 2009). Activation of PAR2 induces assembly of a MAPK signalling cascade by a mechanism that depends on β-arrestins (DeFea et al., 2000). Beta-arrestin dependent endocytosis of PAR2 is required for intracellular targeting of activated ERK1/2, which is retained in the cytosol instead to be translocated to the nucleus. The concentration of ERK1/2 in cytosol depends on the formation of

a signalling complex that includes internalized PAR2, β-arrestin 1, Raf-1 and pERK1/2 (DeFea et al., 2000).
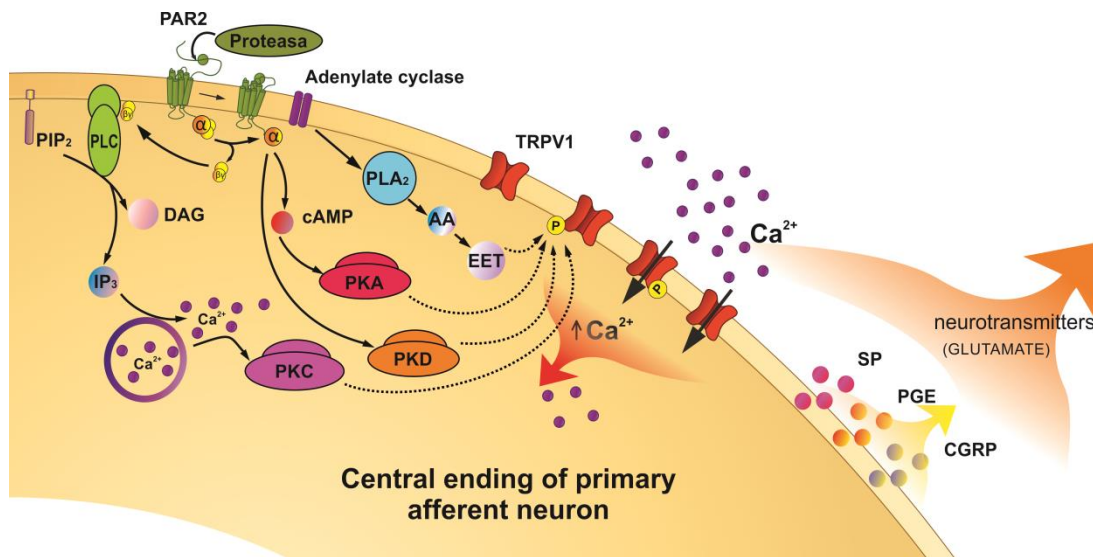
Figure 8. Proposed pathways for PAR2 activation phosphorylated TRPV1 Following inflammation or injury, proteases from multiple cellular sources can cleave PAR2 on primary afferent nerve terminals (DRG nerve ending). Following PAR2 cleavage, G protein signalling mechanisms lead to the production of second messengers and activation of the TRPV1 ion channel. This includes PLC-dependent PIP_2 hydrolysis into diacylglycerol (DAG) and IP3 to trigger Ca^{2+} mobilization from intracellular stores, PLA2 mediated breakdown of phospholipids into arachidonic acid (AA). PKC phosphorylation of Ser824 and PKA activity also contributes to TRPV1. These result in enhanced influx of Ca^{2+} ions and elevated release of prostaglandin E (PGE) CGRP and SP, witch result in enhanced transmission of nociceptive signals (Poole et al., 2013).

2.7.7 Transient receptor potential channels sensitization by PAR2

PAR2 is highly co-expressed with TRPV1 receptors in the DRG neurons (Amadesi et al., 2004, Hoogerwerf et al., 2004). PAR2 activation leads to TRPV1 sensitization via PLC (Amadesi et al., 2004) and via two key kinases PKCε and PKA that phosporylate TRPV1 receptor (Amadesi et al., 2006, Spicarova and Palecek, 2008). Sensitized TRPV1 receptor could be subsequently activated by endogenous agonists (Spicarova and Palecek, 2009, Spicarova et al., 2014b). PAR2 activation

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enhanced TRPV1 agonist capsaicin stimulated neuropeptides (SP, CGRP) release within the spinal cord dorsal horn (Amadesi et al., 2004). *In vivo*, intraplantar injection of PAR2 agonist induced thermal hyperalgesia dependent on TRPV1 receptor activation (Amadesi et al., 2004). TRPV1 mediated DRG and spinal neurons activation in paclitaxel inducted neuropathic pain (Li et al., 2015) It was also shown that spinal TRPV1 activation mediates CCL2 or plantar incision-induced hyperalgesia (Spicarova et al., 2014a, Uchytilova et al., 2014). Recently it was demonstrated that blocking of spinal PAR2 and TRPV1 receptors attenuated oxaliplatin-induced neuropathic pain and this effect was mediated by decreased release of SP and CGRP in the superficial dorsal horn of the spinal cord (Chen et al., 2015).

PAR2 co-expression in rat DRG neurons with TRPV4 receptors and neuropeptides SP and CGRP was also demonstrated (Grant et al., 2007, Poole et al., 2013). Activation of PAR2 sensitized TRPV4 receptors to agonist application due to PLC-β, PIP2, IP3, PLA, PKA, PKC and AA activation (Grant et al., 2007, Poole et al., 2013). These authors also showed that TRPV4 activation promoted SP and CGRP release from afferent nerves in the spinal cord and this process was enhanced by PAR2 agonist pretreatment. *In vivo*, intraplantar injection of PAR2 agonist resulted in mechanical hyperalgesia that was prevented in TRPV4 knock-out mice. The same PAR2 agonist treatment robustly enhanced hyperalgesia induced by the injection of TRPV4 agonist (Grant et al., 2007).

PAR2 is co-expressed with TRPA1 in small DRG neurons (Dai et al., 2007). TRPA1 receptors could be activated by chemical compounds occurring in mustard, wasabi, garlic, onion or cinnamon, they are potential sensors for noxious cold and they are also known for ability to transduce mechanical stimuli (Hill and Schaefer, 2007, Laursen et al., 2014). Electrophysiological studies have shown that PAR2 sensitizes TRPA1, and this effect can be blocked by a PLC inhibitor (Dai et al., 2007). Similarly it was shown that PAR2 activation of PKA and PLC also participate in TRPA1 mediated sensitization in nociceptive transmission (Wang et al., 2008). *In vivo* it has been demonstrated that PAR2 mediates paclitaxel-induced mechanical, heat and cold hypersensitivity through the activation of TRPA1 (Chen et al., 2011).

2.5.8 Role of PAR2 in inflammatory, neuropathic and cancer pain

Critical involvement of PAR2 in the pathogenesis of several types of inflammatory or neuropathic pain was demonstrated previously (Bao et al., 2014a). PAR2 signalling is involved in the sensitization of peripheral nociceptors and dorsal horn neurons in several pathological pain states (Dai et al., 2007, Grant et al., 2007, Chen et al., 2011).

PAR2 are present in many cells involved in inflammation, their activation provokes the release of numerous inflammatory mediators, such as prostaglandins, SP, CGRP or cytokines (interleukine-1 and TNF-α), that in turn may induce or modulate pain perception (Steinhoff et al., 2000, Vergnolle et al., 2001a, Cenac et al., 2002). PAR2-deficient mice developed significantly less pronounced inflammatory hyperalgesia in response to intraplantar injection of formalin or the mast cell degranulator compound 48/80 (Vergnolle et al., 2001a). PAR2 agonist-mediated hyperalgesia was also dependent on a mechanism involving central activation of neurokinin-1 receptors, and release of prostaglandins (Vergnolle et al., 2001a).

Neuropathic pain often develops after peripheral and central nervous system injuries, local inflammation, diabetic neuropathy, viral infection, major surgeries, stroke and after chemotherapy application (Woolf and Mannion, 1999, Ji and Strichartz, 2004). In a chemotherapy (paclitaxel) induced model of neuropathic pain, increased mast cell tryptase activity on the periphery, DRG as well as in spinal cord was demonstrated in mice (Chen et al., 2011). Intrathecal administration of PAR2 antagonist prevented paclitaxel-induced mechanical allodynia and thermal hyperalgesia and attenuated cold hypersensitivity, while it was also selectively blocked by systemic administration of TRPV1, TRPV4 and TRPA1 receptors antagonists. All these TRP receptors were also shown to be sensitized after PAR2 activation, probably due to the activation of PKC and PKA pathways (Chen et al., 2011). It was shown that PAR2 activation may mediate the increased cAMP and PKA activity and cAMP-dependent neuronal hyperexcitability in the model of prolonged compression of DRG. The application of PAR2 antagonist resulted in blocking of the activation of the cAMP-PKA pathway, which contributes to the hyperalgesia (Huang et al., 2012).

Proteases and their receptors may be also involved in cancer pain. Since carcinomas and associated inflammatory cells (mast cells) produce and secrete

proteases during carcinogenesis. PAR2 activation was identified as a novel mechanism of cancer-dependent allodynia which was abolished by serine protease inhibition, diminished by mast cell depletion and it was absent in PAR2-deficient mice (Lam et al., 2012). Further the proportion of neurons that exhibited PAR2-immunoreactivity was also increased in this model (Lam et al., 2012). It was suggested that serine protease inhibitors and PAR2 antagonists may be useful for treatment of cancer pain (Lam and Schmidt, 2010).

In the model of bone cancer pain, induction and persistence of pain behaviour correlated with tumor cell implantation and induced up-regulation of PAR2 in sciatic nerve and DRG. PAR2 knock-out or intrathecal administration of PAR2 antagonist prevented and in same case reversed the bone cancer-related pain behaviour and related neurochemical changes in the DRG and spinal cord dorsal horn (Liu et al., 2013). PAR2 was suggested to be an important mediator for peripheral sensitization of bone cancer pain and inhibiting PAR2 activation as a new therapeutic target in bone cancer pain development and therapy (Liu et al., 2013).

2.6 Pro-inflammatory chemokine CCL2 in nociceptive transmission

Chemokines are a family of small cytokines with chemotactic activity whose main function is to regulate cell migration. It is a low molecular weight (8-10 kDa), structurally very similar peptides that are classified according to common structural characteristics and the presence of four cysteine residues in conserved regions into four categories: CC, CXC, CX3C, and C chemokines (Fernandez and Lolis, 2002). These four groups of chemokines are distinguished not only structurally but also functionally. Each group has the ability to chemotaxis other types of leukocytes.

Under certain pathological conditions glia and immune cells located in the spinal cord may, released proinflammatory cytokines and participating in the modulation of nociceptive signalling. Some chronic pain states induced by injuries of the central and peripheral nervous systems leading to hypertrophy of microglia and astrocytes, which are frequently produce cytokines, chemokines and other pro-nociceptive substances. The mechanisms regulating neuro-glial communication during neuropathic pain are considered very important as they represent a potential source of new therapeutic targets. Especially chemokines are now in the spotlight as they were shown to play a crucial role in mediating the transmission of activity between neurons and glial cells on spinal cord level which leads to increased nociception. The chemokine CCL2 in particular is considered pivotal during neuropathic pain states. The chemokine-mediated augmentation of neuron-microglia communication critically contributes to the amplification of nociceptive transmission that occurs during neuropathic pain. Greater understanding of neuron–microglia interactions during chronic pain states has led to the identification of new therapeutic targets, including chemokines and their receptors.

CCL2, formerly known as monocyte chemoattractant protein-1, and its main receptor CCR2 (Figure 9) was studied and recognized their importance, especially in the process of neuropathic pain development after peripheral nerve injury (Zhang et al., 2007, Zhang et al., 2012). In chronic constriction of the sciatic nerve model was detected upregulation of CCL2 in dorsal root ganglion (DRG) neurons and the superficial dorsal horn (DH) of the spinal cord associated with pain hypersensitivity (Zhang and De Koninck, 2006). The intrathecal application of exogenous CCL2 induced mechanical allodynia and thermal hyperalgesia in rats and mice while CCR2 knockout mice did not show any change in acute pain sensation (Tanaka et al., 2004,

Gao et al., 2009b). And, the development of pain hypersensitivity after peripheral nerve injury was attenuated in these animals (Abbadie et al., 2003).

CCL2 was shown to be present in predominantly small and medium size DRG neurons, co-expressed with TRPV1 receptors (Dansereau et al., 2008). In the spinal cord, CCL2 was detected in the superficial dorsal horn, where is co-localization with SP and CGRP was demonstrated in axon terminals (Dansereau et al., 2008).
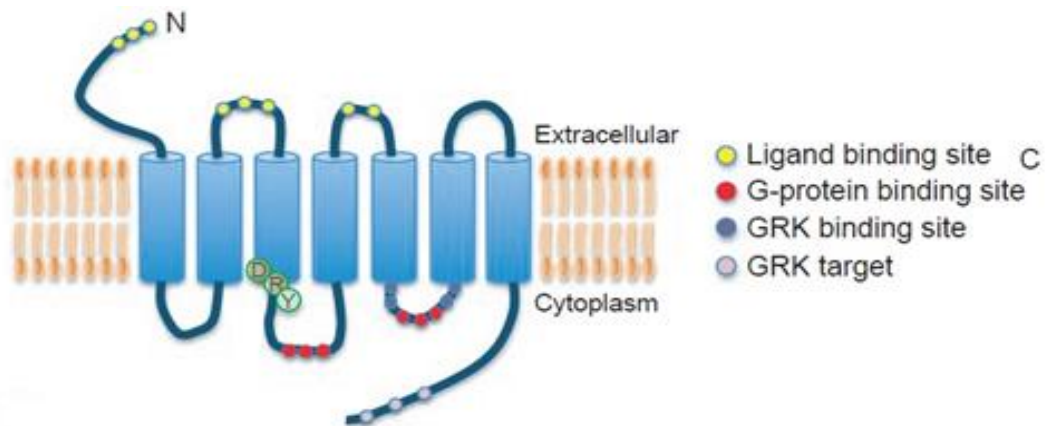
Figure 9. *Schematic diagram of the CCR2 protein: it consists of four extracellular domains, seven transmembrane domains, and four intracellular domains. The DRY (Asp–Arg–Tyr) motif, which is important for G-protein-mediated signal transduction, is located in the juxtamembrane region of the second intracellular loop. G-protein binding sites are also located on the second and third intracellular loops. G-Protein receptor kinase (GRK) binds to the third intracellular loop and phosphorylates the GRK targets on the C-tail to initiate internalization of the CCR2 receptor after ligand binding. Adapted and adjusted from (Yamasaki et al., 2011)*

Peripheral nerve injury triggered CCL2 release from the central endings of primary nociceptive neurons in the DH and act as a neuromodulator (Thacker et al., 2009). CCR2 receptors are expressed and co-localized with TTX- resistant sodium channel NaV1.8 in DRG neurons of naïve rats (Bhangoo et al., 2007). And under pathological conditions the up-regulation of CCR2 receptors expression was clearly demonstrated on the small DRG neurons with TRPV1 receptor-expressing, and also on larger neurons (Jung et al., 2008, Fu et al., 2010).

In the spinal cord, CCR2 receptor expression was found in dorsal horn neurons and astrocytes, their presence in microglia is still not concluded (Abbadie et al., 2003, Knerlich-Lukoschus et al., 2008, Gao et al., 2009a, Old and Malcangio,

2012). The application of the chemokine CCL2 was shown to potentiate responses to capsaicin in experiments with HEK (human embryonic kidney) cells transfected with CCR2 and TRPV1 receptors (Jung et al., 2008). CCL2-induced TRPV1 receptor sensitization (Figure 10) was completely blocked by PKC and PLC inhibitors (Jung et al., 2008).

Pre-treatment of DRG neuronal cultures with CCL2 increased the density of capsaicin-evoked currents and up-regulated TRPV1 receptor mRNA, in dependence on the activation of the phosphatidylinositol-3 kinase (PI3K) pathway (Kao et al., 2012).
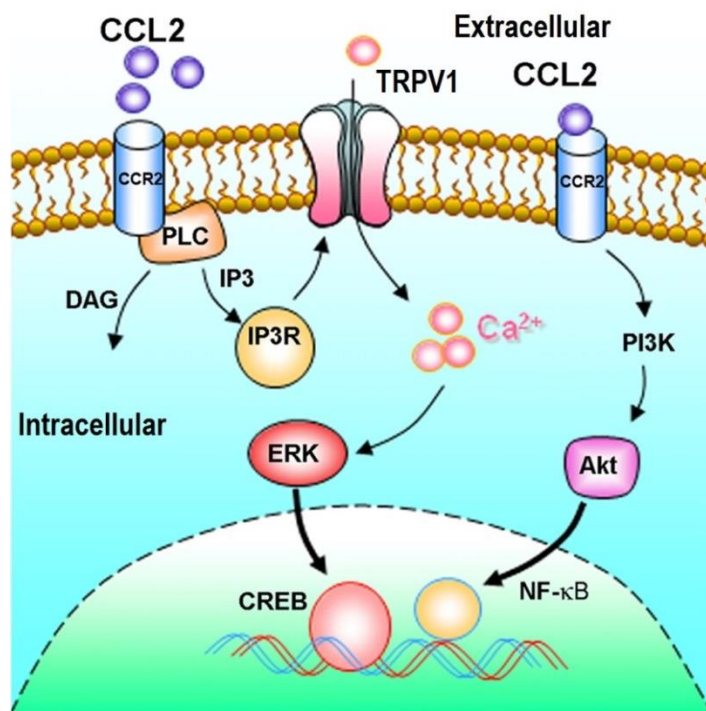
Figure 10. Schematic illustration demonstrating signalling pathways involved in CCL2-mediated neuroprotection in rat primary neurons. CCL2 binding to its cognate CCR2 receptor stimulates the PLC/IP3R pathway, which in turn activates TRPV1 channels, resulting in elevation of $[Ca^{2+}]i$ transients. $[Ca^{2+}]i$ elevation then results in activation of MEK/ERK pathways, leading to CREB activation and consequently neuronal survival. In addition to this, CCL2 binding to CCR2 can also activate another distinct pathway, PI3K/Akt/NF-κB, which can also lead to potentiation of neuronal survival. Adapted and adjusted from (Yao et al., 2009)

2.7 Paclitaxel and the role of TRPV1 and TLR4 receptors in the spinal cord

Toll-like receptors (TLRs) are another important member of the receptors involved in pain transmission at the spinal cord level, especially in acute and chronic itch and pain (Christianson et al., 2011). TLR4 is widely expressed in the glial cells and primary sensory neurons to sense exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) released by tissues after injury or cellular stress (Bsibsi et al., 2002). It is also known that stimulation of TLR4 initiates a series of signalling cascades that result in the activation of nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs) to induce the release of pro-inflammatory cytokines such as, TNF-α and IL-1β (Stokes et al., 2013). It was demonstrated that TLR are expressed on primary sensory neurons with TRPV1 (Liu et al., 2010, Liu et al., 2012). Particularly TLR4 plays a key role in the transition of inflammatory pain to chronic status and in promoting the generation of nerve injury pain (Christianson et al., 2011). More recently, the expression and signalling of TLR4 was shown to be increased with paclitaxel treatment and co-treatment of rats with a TLR4 antagonist during chemotherapy prevented the development of the chemotherapy-induced peripheral neuropathy (CIPN) phenotype (Li et al., 2014).

Paclitaxel is the frontline chemotherapeutic agent used to treat many of the most common solid tumors (Hagiwara and Sunada, 2004). Peripheral neuropathy is the major dose-limiting adverse effect of paclitaxel and can force dose reduction or even discontinuation of therapy, thus affecting survival (Chaudhry et al., 1994) Moreover, CIPN often becomes a chronic condition that is refractory to treatment, affecting rehabilitation, productivity, and quality of life in cancer survivors (Boyette-Davis et al., 2011, Boyette-Davis et al., 2013, Kosturakis et al., 2014).

Paclitaxel appears to engage TLR4 in the same fashion as its canonical, well known pro-inflammatory agonist lipopolysaccharide (LPS) (Han et al., 1994, Li et al., 2013), resulting in the induction and release of the same pro-inflammatory cytokines from macrophages and other cells (O'Brien et al., 1995). The same signalling cascade (Figure 11) as in macrophages appears to be activated by paclitaxel in peripheral sensory neurons, resulting in downstream activation of the TLR4 adapter proteins MyD88-nuclear factor-κB (NF-κB) and

TIR-domain-containing adapter-inducing interferon-β (TRIF) and the same mitogen-activated protein kinase (MAPK) signalling cascades (Li et al., 2015).

TLR4 and TRPV1 were co-localized in human trigeminal ganglion neurons and shown to underlie infection-induced oral pain (Diogenes et al., 2011).

In addition to chronic neuropathy, paclitaxel is also associated with an acute pain syndrome (Reeves et al., 2012). Like chronic CIPN, the paclitaxel acute pain syndrome is associated with the development of overt neuropathy and is characterized by patient complaints of deep muscle aches and arthralgia (Reeves et al., 2012). Also like chronic CIPN, there is no clear preventative treatment and the underlying mechanisms remain poorly defined.
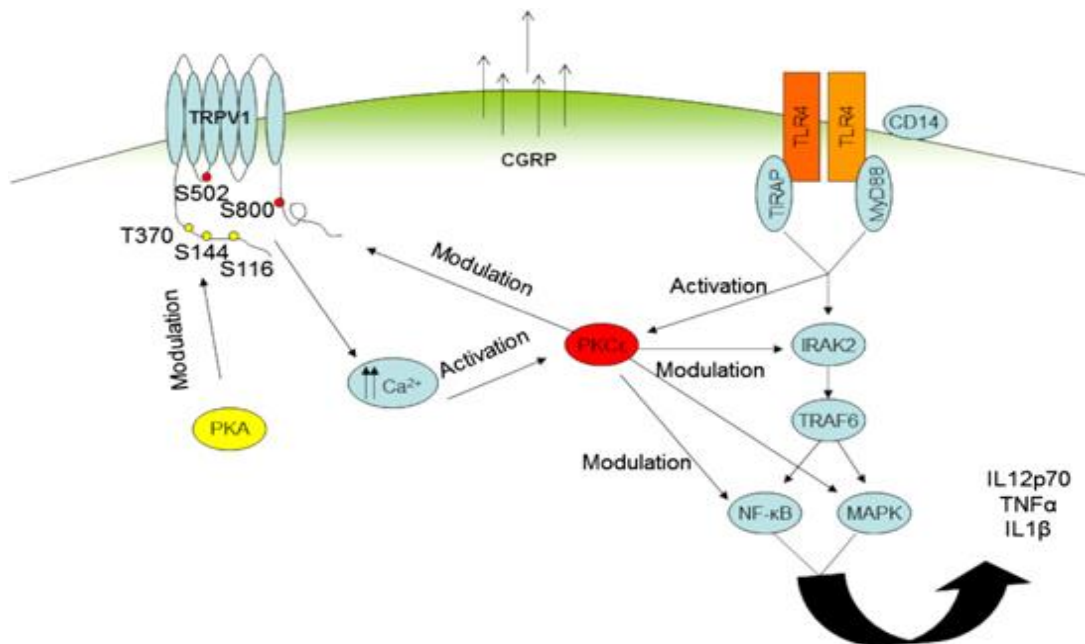
Figure 11. After TLR4 activation by LPS (paclitaxel) an intracellular cascades are initiates leading to the downstream activation of NF-κB and MAPK and the production of cytokines. TLR4 activates PKCε through TIRAP and MyD88 that can potentially bind to TRPV1 via S800- and S502-binding sites. This results in the release of the modulatory neuropeptide CGRP. PKA have also the ability to bind to TRPV1. In addition, Ca^{2+} can activate PKCε for further modulation. Adapted and adjusted from (Assas et al., 2014).

3. AIMS OF THE THESIS

In my work I have focused to study mechanisms of nociceptive signalling at the spinal cord level. I was especially interested in modulation of synaptic transmission in the superficial dorsal horn of spinal cord at the first synapse in the nociceptive pathway. The synapse between the primary afferent fibers and dorsal horn neurons constitutes one of the most important locations in regulation of nociceptive transmission. The main interest was on the interaction of TRPV1, PAR2, CCL2 and TLR4 in this process.

- Study the effect of PAR2 activation in the spinal cord in behavioural experiments

- Investigate the involvement of PAR2 in the transmission and modulation of nociceptive signalling spinal cord dorsal horn neurons using electrophysiological recording from spinal cord slices.

- Examine the role of CCL2 and TRPV1 receptors in nociceptive signalling and modulation at spinal cord level.

- Research the role of TRPV1 and TLR4 receptors in the chemotherapy (paclitaxel) induced neuropathy.

4. METHODS

4.1 Statement of ethical considerations

All experiments were approved by the Animal Care and Use Committee of the Institute of Physiology CAS and were carried out in accordance with the guidelines of the International Association for the Study of Pain, the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, and EU Directive 2010/63/EU for animal experiments. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilise alternatives to in vivo techniques, if available.

4.2 Animal care and utilization

Altogether 71 male Wistar rats (Institute of Physiology CAS breeding programme with government permission for breeding laboratory animals) were used in this study, 43 of them (P21 - P23) were used for electrophysiological recording and 28 adult rats older than 3 months with a minimum weight of 250 g were used for behavioural testing. The animals were housed in a temperature-controlled facility at $23 \pm 2°C$ with free access to food and water and maintained on a 12 h light, 12 h dark cycle and were checked twice a day. All the animals were handled only for a necessary period of time and throughout the experiment did not show any signs of stress or illness. Animals were sacrificed at the end of the experiment by deep anaesthesia with 4 % isoflurane (Forane®, Abbott) and subsequent medulla interruption.

4.3 Spinal cord slice preparation

Acute spinal cord slices were prepared from male Wistar rats on postnatal days P21 - P23, similar to previously published data (Spicarova and Palecek, 2009). After deep anaesthesia with 4 % isoflurane (Forane®, Abbott), the lumbar spinal cord was removed and immersed in oxygenated ice-cold dissection solution containing (in mM): 95 NaCl, 1.8 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, 50 sucrose. The spinal cord was then fixed to vibratome stage (Leica VT1200S, Germany) using cyanoacrylate glue in a groove between two agar blocks. Transverse slices 300 μm thick were cut from the lumbar segment L3-L5, incubated in the dissection solution for 30 min at 33 °C and then stored in a recording solution at room temperature until used for the electrophysiological experiments. The

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recording solution contained (in mM): 127 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 25 D-glucose. For the actual measurement, slices were transferred into a recording chamber continuously perfused with the recording solution at a rate ~ 2 ml/min. All extracellular solutions were saturated with carbogen (95 % O_2, 5 % CO_2) during the whole process.

4.4 Patch-clamp recordings

Patch-clamp recordings were made in acute spinal cord slices from superficial dorsal horn neurons laminae I and II_{outer} (Figure 12). Individual neurons were visualized using a differential interference contrast (DIC) microscope (Leica, DM LFSA, Germany) equipped with an near infrared-sensitive camera (Hitachi KP-200P, Japan) with a standard TV/video monitor. Patch pipettes were pulled from borosilicate glass tubing with resistances of 3.5 - 6.0 MΩ when filled with intracellular solution. The intracellular pipette solution contained (in mM): 125 gluconic acid lactone, 15 CsCl, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 MgATP, 0.5 NaGTP and was adjusted to pH 7.2 with CsOH. Voltage-clamp recordings in the whole-cell configuration were performed with an Axopatch 200B amplifier and Digidata 1440A digitizer (Molecular Devices, USA) at room temperature (~ 23 °C). Whole-cell recordings were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. The series resistance of neurons was routinely compensated by 80 % and was monitored during whole experiment. AMPA receptor-mediated spontaneous, miniature and evoked EPSCs were recorded from neurons clamped at -70 mV in the presence of 10 μM bicuculline and 5 μM strychnine. Miniature EPSCs were distinguished by the addition of 0.5 μM tetrodotoxin (TTX) to the bath solution. In order to record evoked EPSCs, a dorsal root was stimulated using a suction electrode with glass pipette filled with an extracellular solution using a constant current isolated stimulator (Digitimer DS3, England). The intensity of the stimulation was adjusted to evoke stable EPSC with 0.5 ms stimulus duration and at least 3× the minimal stimulus current at a frequency of 0.033 Hz.

The experiments started with control recordings (4 min), followed by PAR2 agonist (SLIGKV-NH₂, 100 μM, 4 min) application. In the groups where antagonist was used (SB 366791, 10 μM; staurosporine, 250 nM), it was applied for 4 min after the control recording as a pre-treatment and then with SLIGKV-NH₂ (100 μM) as co-application. Concentration of SB 366791 used for experiments was determined

from $IC_{50} = 7.5 \pm 1.8$ nM (Leszkiewicz et al., 2000) and our earlier studies (Spicarova and Palecek, 2009, Spicarova et al., 2014a), concentration of SLIGKV-NH_2 was based on the EC_{50} (~ 1 µM) (Kawabata et al., 2004) and previously used effective concentrations (Huang et al., 2011). The mEPSC and sEPSC activity was always evaluated during the last two minutes of the specific application. The evoked EPSCs were recorded every 30 s, the average amplitude of 4 evoked currents in the last two minutes of the particular application was always used for evaluation of the specific condition. Neurons with capsaicin-sensitive afferent input were identified by an increase of EPSC frequency (> 20 %), measured after capsaicin (200 nM) application at the end of each recording protocol.

The concentration of the CCL2 (10nM) solution applied in our experiments was defined according to the effective concentration used previously (Gao et al., 2009b). Concentration of the TRPV1 receptors antagonist SB366791 was set based on our and other published data (Gunthorpe et al., 2004, Ferrini et al., 2007, Spicarova and Palecek, 2009). For experiments stock solution of CCL2 (R&Dsystems, Minneapolis, MN) was prepared using 0.1% BSA (Sigma-Aldrich) and paclitaxel (50 nM) (TEVA Pharmaceuticals) was prepared using 99.9 % DMSO.
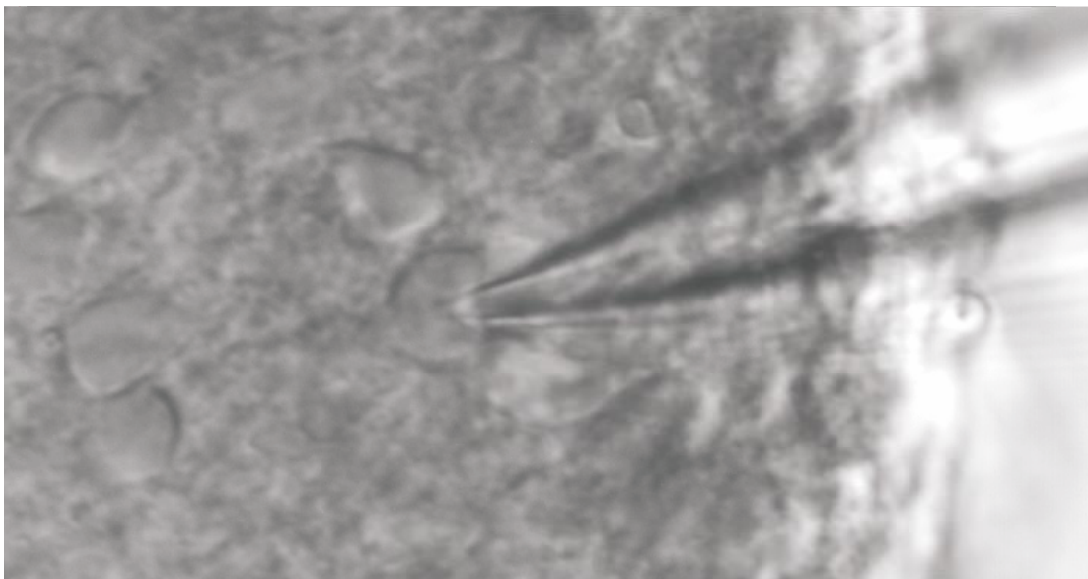

Figure 12. Superficial dorsal horn neuron patched in acute spinal cord slice.

Software package pCLAMP 10 (Molecular devices, USA) was used for data acquisition and subsequent off-line analysis. Data segments of 2 min duration were analysed for each experimental condition. Only EPSCs with an amplitude of 5 pA or greater (which corresponded to at least twice the recording noise level) were included in the frequency analysis. The same events and data segments were used for amplitude analysis. Data are expressed as mean ± standard error of the mean (SEM). Data were normalized as a percentage of the control value (100 %). For statistical analysis of significant differences One Way ANOVA or One Way repeated measures ANOVA were used followed by Holm-Sidak post hoc test. A Kolmogorov-Smirnov test was used to evaluate statistical significance for cumulative data.

4.5 Drug treatment

All basic chemicals, used for the preparation of the dissection, recording and intracellular solution, were of analytical grade and purchased from Sigma-Aldrich (Prague, Czech Republic) and Tocris Bioscience (Bristol, UK). Capsaicin, SLIGKV-NH$_2$, VKGILS-NH$_2$, SB 366791 and staurosporine were dissolved in DMSO, which had a concentration of < 0.1 % in the final solution.

4.6 Intrathecal catheter implantation

Experiments were conducted using adult male Wistar rats (250 - 300 g). Lumbosacral catheters were implanted between the L4 - L5 vertebrae one week before the experiment. Catheter implantations were performed under brief isoflurane (3 %, Forane®, Abbott), followed by ketamine (100 mg/kg) and xylazine (16 mg/kg) anaesthesia. The catheters were constructed from polyethylene tubing (PE5) and were fixed with dental cement (Duracryl) to the vertebral bones. The other end of each catheter was fixed to PE10 tubing and externalized on the back of the animal. The positions of the catheters were verified by a dye injection at the end of each experiment. Intrathecal drugs were applied and flushed (45 or 50 μl) from the catheter by physiological solution: SLIGKV-NH$_2$ and VKGILS-NH$_2$ (10 μl, 8 μg), SB 366791 (15 μl, 0.43 μg), staurosporine (15 μl, 0.014 μg), CCL2 (10 μl, 1.4 μg).

4.7 Behavioural tests

Experiments were conducted on rats, previously implanted with intrathecal catheter, kept in plastic cages with soft bedding, with free access to food and water and maintained on a 12 h light, 12 h dark cycle. The paw withdrawal latency (PWL)

to thermal stimulation was tested using a plantar test apparatus (Ugo Basile, Italy) with radiant heat applied to the plantar surface of each hindpaw. Rats were placed in nonbinding, clear plastic cages on a clear glass plate, elevated to allow application of controlled heat source underneath. Each rat was left to adapt to the testing environment for at least 15 min prior to any stimulation. The hindpaw withdrawal latencies were measured automatically with the apparatus. Each hindpaw was tested 4 times with at least 5 min between the trials. Baseline withdrawal latencies were determined in all animals before any experimental procedure.

The paw withdrawal threshold (PWT) to tactile stimulation was tested manually with an electronic von Frey device (IITC Life Science, Model 2390 Series) where a probe tip was applied to the plantar surface of each hindpaw. The PWT was defined as the force (mN) that evoked an active paw withdrawal response. Each paw was tested 4 times at each time interval and the mean was calculated. The averaged values from the left and right hind paws in individual animals were then averaged in the experimental groups. All data are expressed as mean ± SEM. One Way repeated measure ANOVA with Bonferroni post hoc test and Two-Way ANOVA with Student Newman Keuls test was used to identify statistically significant differences.

5. RESULTS

5.1 The effect of spinal PAR2 activation for thermal and mechanical sensitivity in naive animals

The role of spinal PAR2 receptors in thermal and mechanical hypersensitivity was investigated in behavioural experiments. The concentration of PAR2 activating peptide, SLIGKV-NH$_2$, was based on effective doses used in previous studies (Alier et al., 2008, Huang et al., 2011). Intrathecal administration of SLIGKV-NH$_2$ (8 μg in 10 μl of saline) decreased the paw withdrawal latency in response to a thermal stimulus already one hour after the treatment (81.6 ± 4.9 %, n = 7, p < 0.001, Figure 13 A). This decrease of PWLs lasted and was even more pronounced at 4 h after the SLIGKV-NH$_2$ administration (76.1 ± 3.5 %, p < 0.001). The PWL returned close to the control pre-treatment values at 24 h after the SLIGKV-NH$_2$ administration (96.3 ± 0.8 %).

In control experiments a non-active reverse peptide VKGILS-NH$_2$ was used. Intrathecal administration of VKGILS-NH$_2$ (8 μg in10 μl of saline) did not change the PWL at any of the tested time points (1 h, 99.5 ± 1.3 %; 2 h, 99.3 ± 2.6 %; 4 h, 97.6 ± 1.8 %; 24 h, 99.6 ± 1.1 %; n = 6, Figure 13 A). Another set of behavioural experiments was performed to test the role of spinal TRPV1 receptors in hyperalgesia induced by spinal PAR2 activation. Intrathecal administration of TRPV1 antagonist SB 366791 (0.43 μg in 15 μl of saline, n = 6) 5 min before the SLIGKV-NH$_2$ (8 μg in 10 μl of saline) treatment prevented any significant change from the control values (1 h, 103.4 ± 5.3 %; 2 h, 93.2 ± 4.3 %; 4 h, 100.0 ± 3.6 %; 24 h, 93.4 ± 3.6 %, Figure 13 A). Pre-treatment with SB 366791 thus completely abolished the thermal hyperalgesia induced by the SLIGKV-NH$_2$ application alone.

The involvement of PKs activation in PAR2-induced hyperalgesia was investigated next. A broad spectrum PKs inhibitor staurosporine (0.014 μg in 15 μl of saline, n = 7) was administered 5 min before SLIGKV-NH$_2$ (8 μg in 10 μl of saline). Paw withdrawal latencies were slightly decreased after this treatment, while only at 2 h and at 4 h intervals it reached a statistical significance (1 h, 91.8 ± 6.1 %; 2 h, 85.2 ± 7.4 %, p < 0.05; 4 h, 85.5 ± 6.2 %, p < 0.05; 24 h, 99.5 ± 2.0 %,

Figure 13 A). Our results indicate that inhibition of spinal PKs significantly attenuated the PAR2-induced thermal hyperalgesia.

Tests of mechanical sensitivity, performed at the same time, did not show any effect after i.t. application of any of the tested drugs (SLIGKV-NH$_2$, VKGILS-NH$_2$, SB 366791, staurosporine, Figure 13 B). These results suggest that activation of spinal PAR2 failed to change mechanical sensitivity at any of the tested time points.
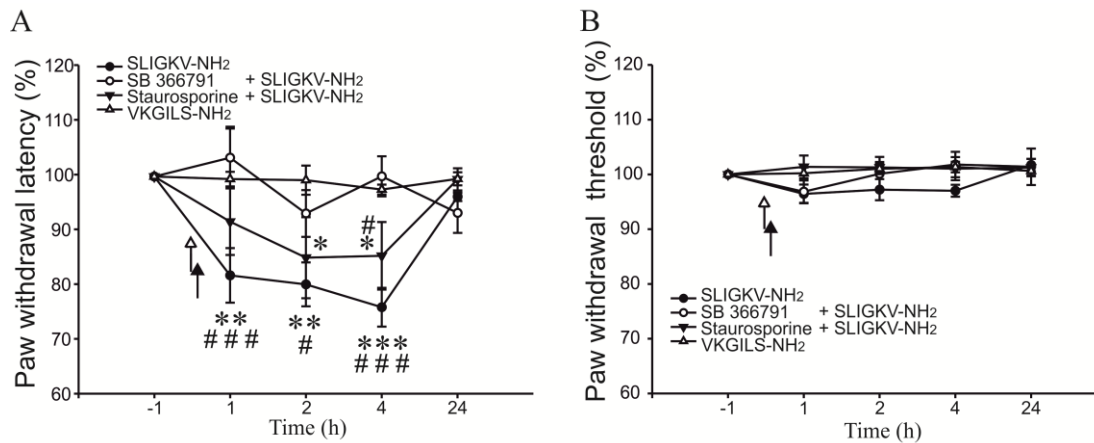
Figure 13. *Activation of spinal PAR2 induced thermal hyperalgesia.* **(A)** *Intrathecal administration of PAR2 activating peptide SLIGKV-NH$_2$ (8 µg, 10 µl, n = 7) decreased the PWLs to radiant heat stimulation for several hours after the treatment. An inactive reverse peptide VKGILS-NH$_2$ (8 µg, 10 µl, n = 6) did not change the thermal threshold. TRPV1 antagonist SB 366791 (0.43 µg, 15 µl, n = 6) pre-treatment prevented SLIGKV-NH$_2$ induced decrease of PWLs. Staurosporine (0.014 µg, 15 µl, n = 7) pre-treatment also partially blocked the PWL decrease induced by SLIGKV-NH$_2$. ↑Application of SB 366791 or staurosporine, ↑ Application of SLIGKV-NH$_2$ or VKGILS-NH$_2$* **(B)** *Paw withdrawal threshold to mechanical stimulation with von Frey filament was not significantly affected by any of the intrathecal treatments. Statistical differences between variously treated groups were identified using Two-way ANOVA followed by multiple comparisons Student Newman Keuls test (* p < 0.05, ** p < 0.01, *** p < 0.001).*

5.2 Modulation of miniature excitatory post synaptic currents (mEPSCs) in spinal cord slices by PAR2 activation

Modulation of mEPSCs activity recorded from superficial dorsal horn neurons after PAR2 activation was tested *in vitro* using spinal cord slices. Miniature EPSCs were recorded in 41 neurons, where the average control mEPSC frequency was 0.81 ± 0.09 Hz. Out of these 41 neurons 38 showed an increase of mEPSC frequency (7.93 ± 1.80 Hz, n = 38, p < 0.001) after TRPV1 agonist capsaicin ($0.2\,\mu M$) application at the end of the recording. This suggests the presence of presynaptic TRPV1 receptors in great majority of the recorded neurons.

Application of SLIGKV-NH$_2$ ($100\,\mu M$, 4 min) significantly decreased the mEPSC frequency to 62.8 ± 4.9 % (n = 17, p < 0.001), when compared to the pre-treatment values (Figure 14 A, B). The inhibitory effect on the mEPSC frequency persisted during the 4 minutes washout period (60.7 ± 5.5 %, p < 0.001). In a set of control experiments inactive peptide VKGILS-NH$_2$ ($100\,\mu M$) did not elicit any changes of mEPSC frequency (99.3 ± 6.6 %, n = 6). Possible interaction of PAR2 and TRPV1 receptors was evaluated next. Application of TRPV1 antagonist SB 366791 ($10\,\mu M$, 4 min) did not change the frequency of mEPSC (103.1 ± 8.3 %, n = 8). Subsequent co-application of SB 366791 ($10\,\mu M$) with SLIGKV-NH$_2$ ($100\,\mu M$, 4 min) also did not change the mEPSC frequency significantly (87.2 ± 10.2 %, n = 8, Figure 14 B), when compared to the period of pre-treatment with SB 366791. These results indicate that application of TRPV1 antagonist prevented the PAR2 activation-induced inhibitory effect on the mEPSC frequency.

The involvement of protein kinases activation in the PAR2-induced inhibitory effect on mEPSC frequency was evaluated in another group of neurons. Application of staurosporine (250 nM, 4 min) alone had no effect on the mEPSC frequency (97.3 ± 15.9 %, n = 10). Subsequent co-application of staurosporine (250 nM) with SLIGKV-NH$_2$ ($100\,\mu M$, 4 min) also did not change the mEPSC frequency (94.9 ± 8.5 %, n = 10, Figure 14 B), when compared to the pretreatment with staurosporine alone.

The mean value of the mEPSC frequency inhibition induced by the PAR2 agonist application alone was significantly different from the changes induced by the combination of PAR2 agonist with TRPV1 and PKs antagonists (Figure 14 B). These

results show that staurosporine and SB 366791 prevented the PAR2 mediated mEPSC frequency inhibition.
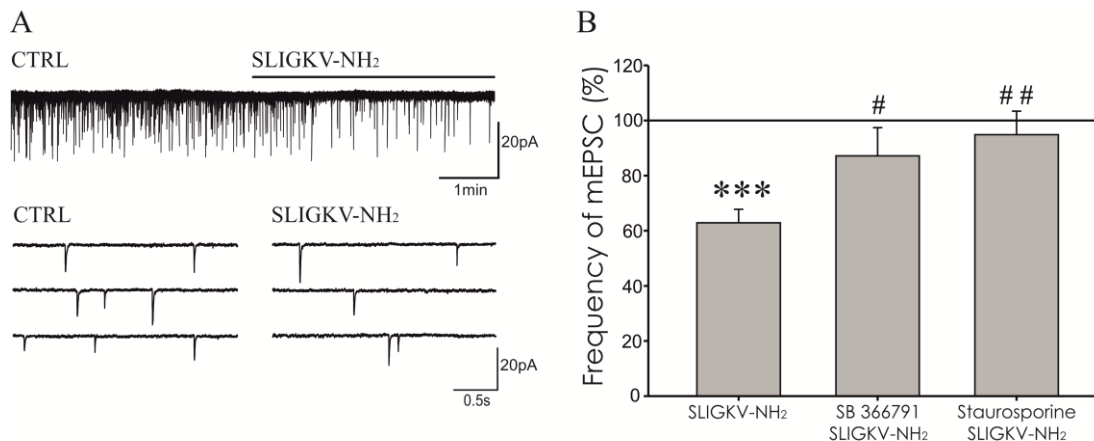
Figure 14. *PAR2 activation decreased the frequency of mEPSCs. **(A)** Application of SLIGKV-NH$_2$ (100 μM, 4 min) lowered the frequency of mEPSC as is documented in the recording from one superficial dorsal horn neuron in acute spinal cord slice. **(B)** Application of SLIGKV-NH$_2$ (100 μM, 4 min) decreased the mEPSC frequency (n = 17; *** p < 0.001) compared to the pretreatment period (100 %). Co-application of TRPV1 antagonist SB 366791 (10 μM, 4 min, n = 8) or staurosporine (250 nM, 4 min, n = 10) prevented the inhibitory effect of SLIGKV-NH$_2$ (100 μM) treatment and the mean mEPSC values were statistically different compared to the application of SLIGKV-NH$_2$ alone ([##] p < 0.01, [###] p < 0.001).*

The average amplitude of the control mEPSCs was 22.7 ± 2.4 pA and did not change significantly during the SLIGKV-NH$_2$ application (21.7 ± 2.1 pA, n = 17, 100 μM) in the first group of neurons. No change of mEPSC amplitude was also present in the cumulative amplitude analysis (Figure 15). Likewise there was no change of mEPSC amplitude in any of the other experimental groups (control 21.9 ± 1.5 pA, VKGILS-NH$_2$ 21.6 ± 1.3 pA, n = 6; control 22.2 ± 1.7 pA, SB 366791 22.4 ± 2.0 pA, SB 366791/SLIGKV-NH$_2$ 21.9 ± 1.6 pA, n = 8; control 22.5 ± 2.5 pA, staurosporine 21.2 ± 2.5 pA, staurosporine/SLIGKV-NH$_2$ 20.9 ± 1.8 pA, n = 10).

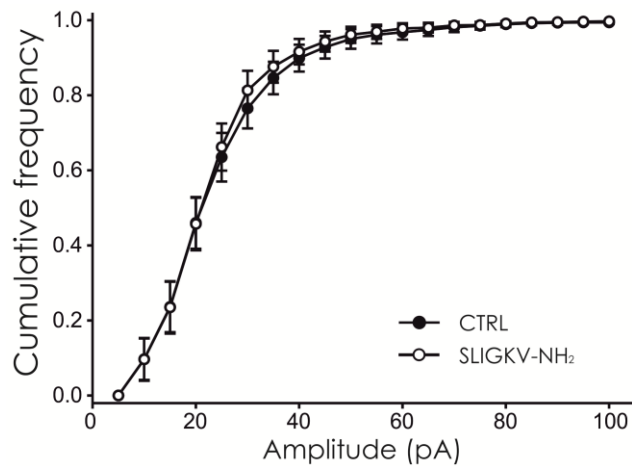***Figure 15.*** *Cumulative amplitude analysis of mEPSCs under control conditions and during application of SLIGKV-NH₂ (100 μM, 4 min, n = 17) did not show statistically significant difference.*

5.3 Modulation of spontaneous excitatory post synaptic currents (sEPSCs) by PAR2 activation in dorsal horn neurons

The effect of PAR2 activating peptide application on spontaneous EPSCs was studied in another group of superficial dorsal horn neurons. In accordance with our previous findings (Spicarova and Palecek, 2009), the basal control sEPSC frequency (1.38 ± 0.7 Hz, n = 41) was significantly higher than the average frequency of mEPSCs recorded in the previous group (0.81 ± 0.09 Hz, n = 41, $p < 0.001$). Out of these 41 neurons, 38 showed sEPSC frequency increase (6.71 ± 1.93 Hz, n = 38, $p < 0.001$) after capsaicin (0.2 μM) application at the end of the experiment.

Bath application of SLIGKV-NH₂ (100 μM, 4 min) significantly increased the sEPSCs frequency to 127.0 ± 5.9 % (n = 16, $p < 0.01$), compared to the pre-treatment values (Figure 16 A, B). The excitatory effect of the SLIGKV-NH₂ application on the sEPSC frequency was slightly diminished, but still persisted during the 4 minutes washout period (118.2 ± 8.0 %, $p < 0.05$). In the group of control experiments, inactive peptide VKGILS-NH₂ (100 μM, 4 min) application did not elicit any change of the sEPSC frequency (94.75 ± 7.93 %, n = 6).

Application of SB 366791 (10 μM, 4 min) did not change the sEPSCs frequency (97.5 ± 9.2 %, n = 10) in another set of experiments. Subsequent

co-application of SB 366791 (10 μM) with SLIGKV-NH$_2$ (100 μM, 4 min) similarly did not change the sEPSCs frequency significantly (106.7 ± 7.9 %, n = 10, Figure 16 B), when compared to the SB 366791 pre-treatment period. These results suggest that SLIGKV-NH$_2$ induced increase of the sEPSC frequency that was mediated by activation of spinal TRPV1 receptors.

Staurosporine (250 nM, 4 min) application had no effect on the sEPSC frequency (90.8 ± 10.1 %, n = 9) in the next experiments. Subsequent co-application of staurosporine (250 nM) with SLIGKV-NH$_2$ (100 μM, 4 min) also did not change the frequency of the sEPSC (108.5 ± 7.2 %, n = 9, Figure 16 B), compared to the pre-treatment period with staurosporine alone. Inhibition of protein kinases thus prevented the PAR2 activation-induced excitatory effect on the sEPSC frequency.

The mean value of sEPSCs frequencies recorded after SLIGKV-NH$_2$ application alone was significantly different from those recorded in the presence of SLIGKV-NH$_2$ with SB 366791 or staurosporine ($^#$ p < 0.05, Figure 16 B). These results suggest that the PAR2 induced increase of sEPSC frequency was at least partially mediated by TRPV1 receptors and protein kinases activation.
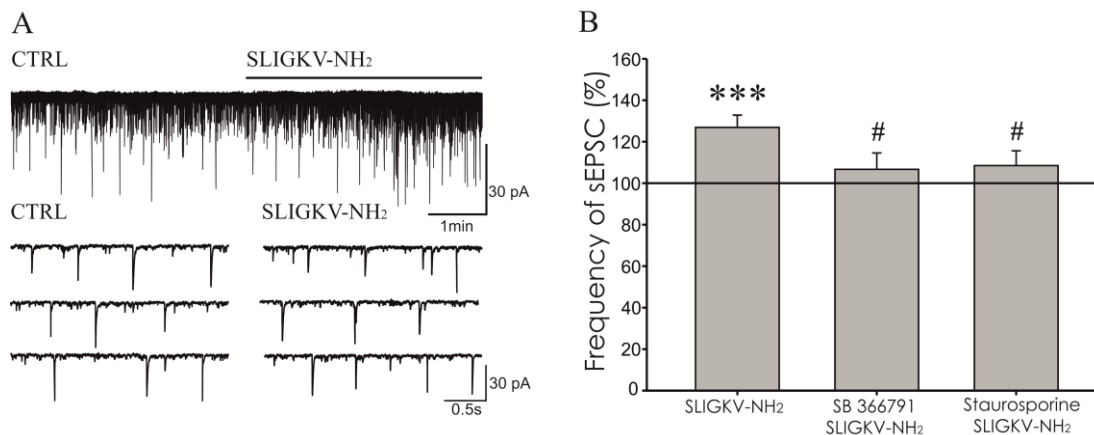
Figure 16. *PAR2 activation increased the frequency of sEPSCs. (A) Application of SLIGKV-NH$_2$ (100 μM, 4 min) increased the sEPSC frequency as documented in recording from one superficial dorsal horn neuron. (B) Application of SLIGKV-NH$_2$ (100 μM, 4 min) increased the sEPSC frequency compared to the pre-treatment values set as 100 % (n = 16; ** p < 0.01). Application of TRPV1 antagonist SB 366791 (10 μM, 4 min, n = 10) or staurosporine (250 nM, 4 min, n = 9) prevented the excitatory effect of SLIGKV-NH$_2$ treatment and the mean sEPSC frequency values were statistically different from the application of SLIGKV-NH$_2$ alone ($^#$ p < 0.05).*

The average amplitude of the recorded sEPSCs did not change significantly in any of the experimental conditions (control 25.7 ± 2.1 pA, SLIGKV-NH$_2$ 25.1 ± 1.9 pA, n = 16; control 25.7 ± 1.2 pA, VKGILS -NH$_2$ 25.9 ± 1.1 pA, n = 6; control 24.6 ± 2.9 pA, SB 366791 23.8 ± 1.2 pA, SB 366791/SLIGKV-NH$_2$ 23.8 ± 1.4 pA, n = 10; control 24.0 ± 1.9 pA, staurosporine 23.6 ± 2.1 pA, staurosporine/SLIGKV-NH$_2$ 24.3 ± 2.1 pA, n = 9). No change of sEPSC amplitude was also detected using cumulative amplitude analysis for the first group of neurons (n = 16, Figure 17).
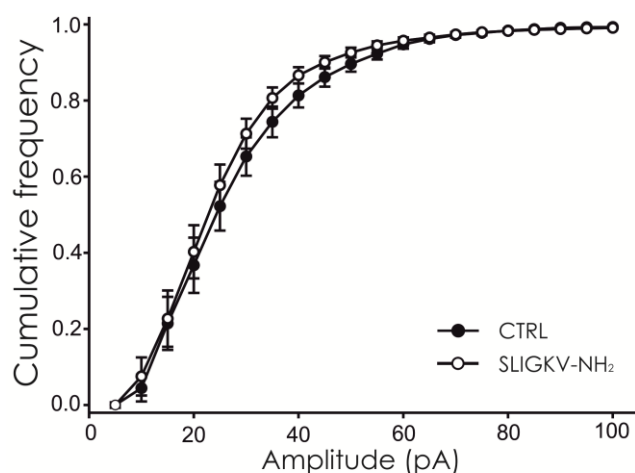
Figure 17. *The amplitude of the sEPSCs did not change during SLIGKV-NH$_2$ application (100 μM, 4 min, n = 16).*

5.4 PAR2 mediated modulation of dorsal root stimulation-evoked EPSCs on spinal cord level

Modulation of eEPSCs by PAR2 activation was tested in superficial dorsal horn neurons, where dorsal root attached to the spinal cord slice was electrically stimulated with glass suction electrode in 30s intervals. Evoked EPSCs were recorded in 41 neurons and 37 of these showed an increase of sEPSC frequency after capsaicin (0.2 μM) application at the end of the experiment.

In the first series of experiments bath application of SLIGKV-NH$_2$ (100 μM, 4 min) increased the amplitude of evoked EPSCs (126.9 ± 12.0 %, n = 17, p < 0.05, Figure 18 A and B). The increase of the eEPSC amplitude was even higher during the 4 min following the SLIGKV-NH$_2$ application (washout period; 148.9 ± 17.7 %,

p < 0.01). Application of the control inactive peptide (VKGILS-NH$_2$, 100 µM, 4 min) did not change the eEPSC amplitude (98.79 ± 12.22 %, n = 6). These results suggest that activation of PAR2 may enhance synaptic transmission in the superficial spinal cord dorsal horn.

Application of SB 366791 (10 µM, 4 min) did not change the amplitude of the eEPSCs (105.5 ± 4.4 %, n = 10). Subsequent co-application of SB 366791 (10 µM) with SLIGKV-NH$_2$ (100 µM, 4 min) also did not change the eEPSC amplitude (98.5 ± 2.5 %, Figure 18 B). The eEPSC amplitude did not change during the washout period as well (101.8 ± 6.8 %). Inhibition of spinal TRPV1 receptors thus prevented the PAR2 activation-induced increase of eEPSC amplitude.

In another group of neurons staurosporine (250 nM, 4 min) application did not change the amplitude of eEPSCs (101.8 ± 4.7 %, n = 9). The subsequent co-application of staurosporine (250 nM) with SLIGKV-NH$_2$ (100 µM, 4 min) similarly had no effect on the eEPSC amplitude (96.9 ± 3.4 %, Figure 18 B). The eEPSC amplitude did not change significantly also during the washout period (91.7 ± 4.8 %). Inhibition of PKs thus prevented the increase of eEPSC amplitude induced by PAR2 activation.
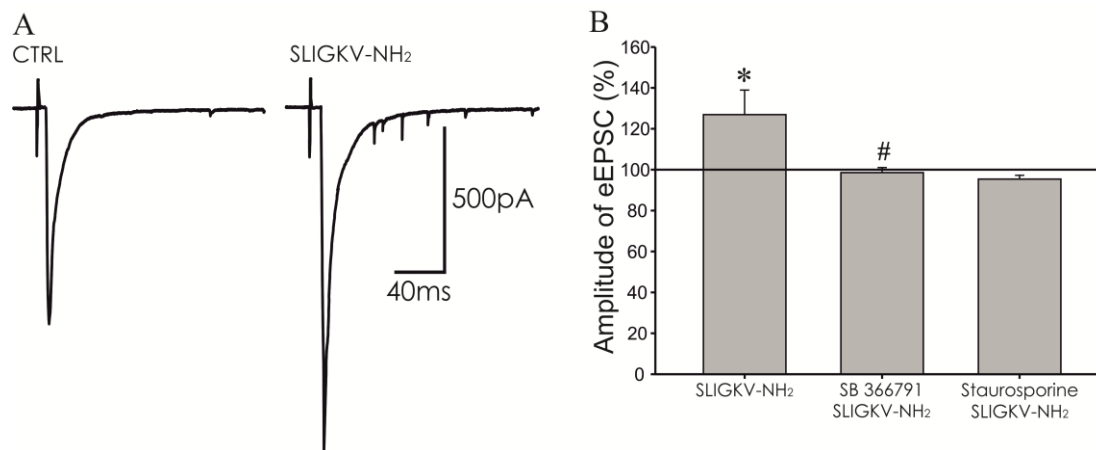
Figure 18. *Activation of PAR2 increased the amplitude of EPSCs evoked by dorsal root stimulation. (A) Application of SLIGKV-NH$_2$ (100 µM, 4 min) increased the amplitude of the evoked EPSC. (B) The increase of eEPSCs amplitude during the SLIGKV-NH$_2$ (100 µM, 4 min) application was statistically significant compared to pre-treatment values (n = 17, ** p < 0.01). Application of SB 366791 (10 µM, 4 min, n = 10) or staurosporine (250 nM, 4 min, n = 9) prevented the SLIGKV-NH$_2$ induced eEPSC amplitude increase and the mean eEPSC frequency values were statistically different from the application of SLIGKV-NH$_2$ alone ([#] p < 0.05).*

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5.5. TRPV1 receptor antagonist prevented the CCL2-induced increase of the eEPSC amplitude

In the next set of experiments, the effect of CCL2 application on the synaptic transmission between the primary afferent fibers and the superficial DH neurons in the spinal cord was studied while using electrical stimulation of the dorsal root in acute spinal cord slices.

The application of CCL2 (10 nM) increased the average amplitude of the eEPSCs to $188.1 \pm 32.1\%$ (n = 18, p < 0.05), when compared to the control values before treatment (100%, Figure 19). The changes in the eEPSC amplitude induced by CCL2 application were not homogenous across the tested population of neurons; 11 of the 18 neurons showed an increase of the eEPSC amplitude to $246.6 \pm 44.4\%$ of the control value (p < 0.05), when an increase above 10% was considered as the dividing criterion. One neuron showed a decrease to 71.2%, and the eEPSC amplitude did not change in 6 neurons ($100.4 \pm 2.2\%$).
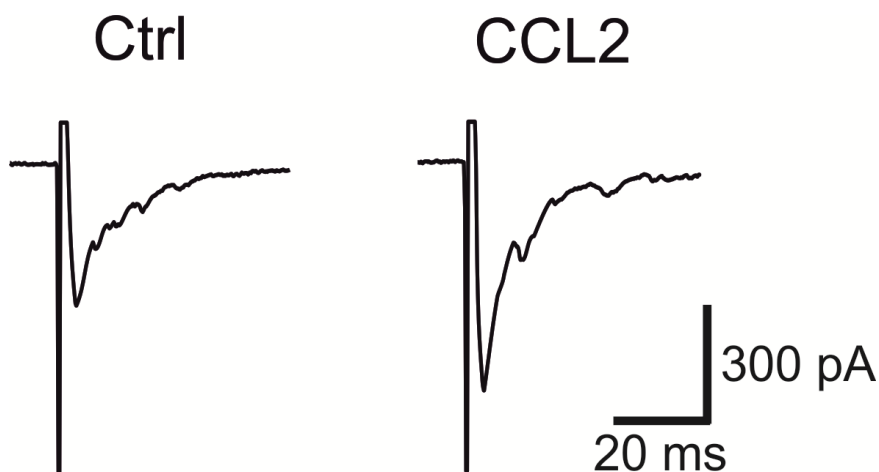
Figure 19. *The application of CCL2 (10 nM) on a spinal cord slice increased the amplitude of the eEPSCs recorded from a superficial DH neuron*

In another group of neurons SB366791 pretreatment (10 µM, 6 min) was tested; such pretreatment did not significantly change the average amplitude of the recorded eEPSCs ($128.1 \pm 17.4\%$, n = 12, p > 0.05 Figure 20 A and B). In this group of neurons, the effect of the co-application of SB366791 (10 µM) with CCL2 (10 nM) was tested as the next step in the experimental protocol (Figure 20 A). The average amplitude of the eEPSCs ($120.8 \pm 17.2\%$, p > 0.05) did not change after SB366791 + CCL2 application compared to the SB366791 pretreatment values.

Out of this population, 5 of the 12 neurons showed an increase (>10% of 168.4 ± 30.8%, P > 0.05), 4 neurons showed a decrease (79.5 ± 4.0%, p > 0.05) and in 3 neurons the eEPSC amplitude did not change (96.6 ± 5.0%). To demonstrate more clearly the effect of SB366791 application and the subsequent SB366791/CCL2 co-application, the mean values of all the treatments were expressed compared to the control values (Figure 20 B). These results indicate that the CCL2-induced increase of the eEPSC amplitude was partially mediated by TRPV1 receptor activation.
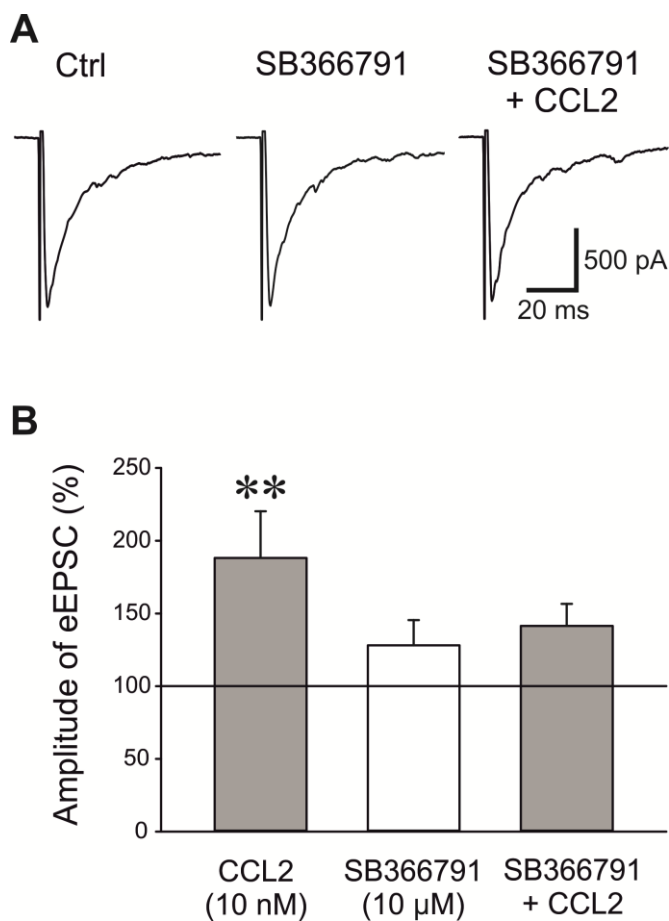
Figure 20. *The CCL2-induced increase of the eEPSC amplitude was diminished by SB366791. (A) The application of the TRPV1 receptor antagonist SB366791 (10 μM) did not change the eEPSC amplitude and prevented its increase following SB366791 (10 μM)/CCL2 (10 nM) co-application. (B) SB366791 pretreatment with subsequent SB366791/CCL2 co-application (n = 12) diminished the CCL2-induced increase of eEPSC amplitude (n = 18). One way ANOVA followed by the Student-Newman-Keuls test was used for statistical analyses; ** p < 0.01.*

5.6 Direct effects of paclitaxel on EPSCs in rat spinal dorsal horn neurons

Acute paclitaxel application (50 nM) in rat spinal cord slices preparation increased mEPSC frequency recorded in superficial dorsal horn neurons compared with the vehicle control (140.7 ± 11.1%; n =14, ***p 0.001, Figure 21 A,C). This effect was blocked when paclitaxel was coapplied with the TRPV1 antagonist SB366791 (10μM), whereas the antagonist alone did not change mEPSC frequency (Figure 21 B). These effects were highly significant across the groups of neurons tested (SB366791 101.5 ± 9.09 %, SB366791+paclitaxel 91.9 ± 5.9 %, n = 10, Figure 21 C). But paclitaxel application did not change the frequency of sEPSCs (104.4 ± 9.0 %; n = 14; P > 0.05, Figure 21 D) and neither the amplitude of EPSCs evoked by dorsal root stimulation (104.7 ± 5.2 % n = 9; P > 0.05, Figure 21 E).
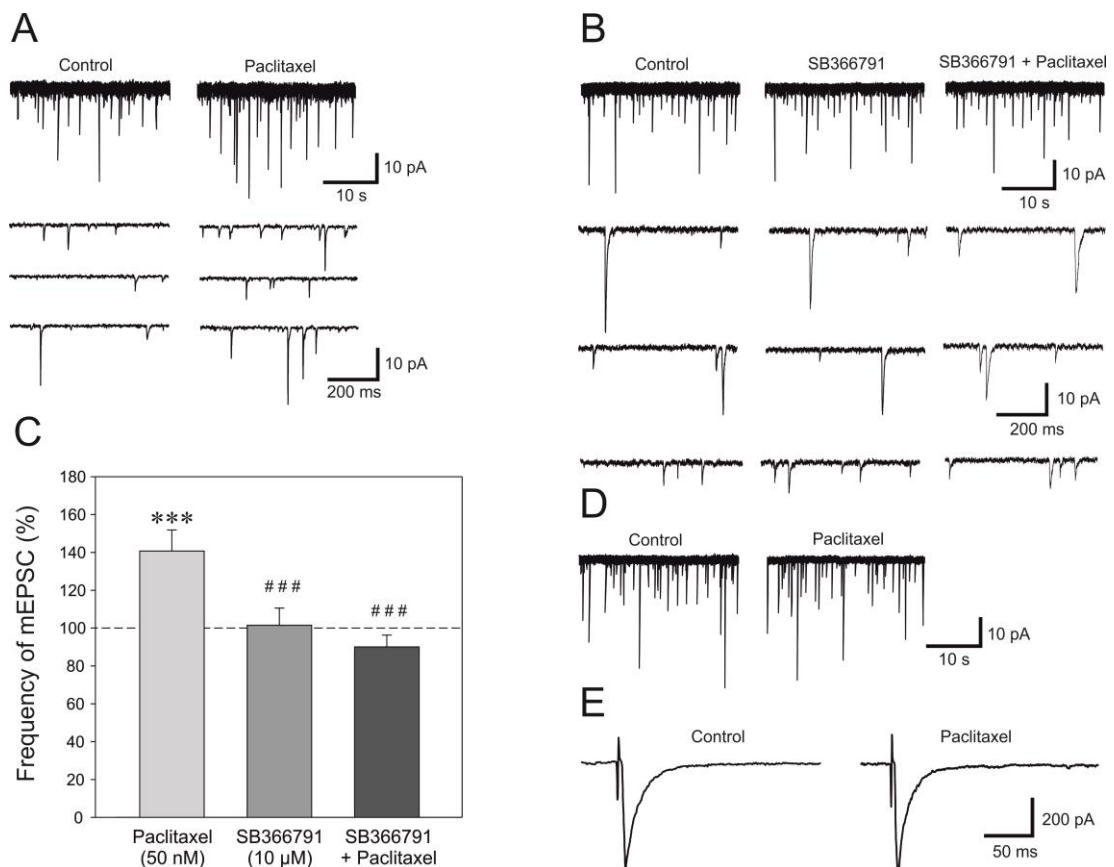
*Figure 21. Paclitaxel application increased mEPSC frequency in superficial dorsal horn neurons in rat spinal cord slice. **A**, Native recording of mEPSC activity before and after paclitaxel (50 nM) application. **B**, The TRPV1 antagonist SB366791 (10 μM) did not change the mEPSC frequency but prevented its increase during co-application with paclitaxel. **C**, Averaged responses demonstrate that paclitaxel*

*treatment induced a significant increase in mEPSC frequency compared with the baseline (control, 100%) value (140.711.1%; n14). This increase was prevented by the TRPV1 antagonist treatment, whereas the antagonist alone had no effect (SB366791 10μM). **D,E** Paclitaxel (50 nM) application did not change the frequency of the sEPSCs or the amplitude of the dorsal root evoked EPSCs. *** p < 0.001 versus control values; ### p < 0.001 versus paclitaxel; one-way ANOVA followed by Student–Newman–Keuls test.*

6. DISCUSSION

Dorsal horn neurons receive nociceptive information from primary afferents that innervate skin and deeper tissues of the body and respond to specific types of noxious and non-noxious stimuli. These afferents fibers terminate in the dorsal horn of spinal cord. The incoming information is processed by complex circuits involving excitatory and inhibitory interneurons, and is transmitted to spinal projection neurons. Synaptic transmission in superficial laminas of dorsal horn is very important for the signal modulation and pain perception. These first synapses of nociceptive pathways allow modulation of the nociceptive information before entering the higher brain centres.

In the following chapters, the results of our studies focused on TRPV1 receptors mediated modulation of spinal cord nociceptive transmission are discussed.

6.1. The role of PAR2 in modulation of nociception

The important role of PAR2 in nociception on the periphery was demonstrated in a variety of pathological pain conditions (Clark et al., 2007, Vergnolle, 2009, Cenac, 2013, Chen et al., 2015, Tillu et al., 2015, Hewitt et al., 2016). The modulation of excitatory synaptic transmission in the superficial dorsal horn of the spinal cord by PAR2 was studied only marginally with different results (Alier et al., 2008, Fujita et al., 2009). In our experiments, we further studied the role of spinal PAR2 activation after intrathecal application of PAR2 and on modulation of nociceptive synaptic transmission in the lamina I and II_o of the dorsal horn.

6.1.1. Effect of PAR2 receptors activation on thermal and mechanical threshold sensitivity after intrathecal application of PAR2 agonist

In our *in vivo* experiments the intrathecal application of PAR2 activating peptide SLIGKV-NH$_2$ induced thermal hyperalgesia in naive adult rats that was prevented by inhibition of spinal TRPV1 receptors and attenuated by inhibition of protein kinases. However, sensitivity to mechanical stimuli did not change in the same experiments. Our results suggest that activation of spinal PAR2 leads to strengthening of nociceptive excitatory synaptic transmission at the spinal cord and

thermal hyperalgesia that is at least partially mediated by TRPV1 receptors activation.

Our results indicated presence of several hours lasting thermal hyperalgesia after intrathecal administration of PAR2 activating peptide SLIGKV-NH$_2$, which corresponds to the earlier findings (Alier et al., 2008). However, this treatment failed to induce mechanical allodynia, which was previously shown after intrathecal application of another PAR2 activating peptide SLIGRL-NH$_2$ (Alier et al., 2008, Huang et al., 2011). This activating peptide was formerly considered as a specific PAR2 agonist, but recently activation of several Mrgprs (Mas-related G-protein-coupled receptors) that induce itch in mice was demonstrated (Han et al., 2013, Namer and Reeh, 2013). Nevertheless SLIGRL-NH$_2$ induced mechanical hypersensitivity was absents in PAR2 knock-out mice (Alier et al., 2008) thus the differences in experimental results pointing rather to the dissimilar experimental approaches and conditions and/or mechanisms in different animal species than the specificity of PAR2 activating peptides. Our results indicate that under control conditions, activation of spinal PAR2 leads preferentially to thermal hypersensitivity. This may be changed under pathological conditions, like bone cancer-evoked pain, when PAR2 are overexpressed predominantly in medium and large DRG neurons (Bao et al., 2015), which could underlie the development of mechanical hypersensitivity. Thermal hyperalgesia induced in our experiments by activation of spinal PAR2 was prevented by inhibition of spinal TRPV1 receptors. In naïve animals the thresholds for thermal and tactile stimuli in peripheral nerves endings are suggested to be unchanged when PAR2 activating peptide is injected intrathecally. It is plausible that the action potentials generated in DRG neurons by the presence of thermal stimulus in the rat paw induced thermal hyperalgesia, which was mediated by sensitization of spinal endings of these afferent neurons by activation of spinal PAR2 and TRPV1 receptors. In this regard the mechanical hypersensitivity was not mediated by these central terminals coexpressing both PAR2 and TRPV1. The attenuation of spinal inhibitory synaptic transmission by PAR2 demonstrated by the reduced frequency and amplitude of sIPSCs in the spinal cord dorsal horn (Huang et al., 2011) may also contribute to the hypersensitivity development. The mechanism involving TRPV1 activation in PAR2-induced hyperalgesia was demonstrated also after the activation of peripherally localized PAR2 (Amadesi et al., 2004) and this corresponds well to TRPV1 mediated thermal hypersensitivity (Caterina et al., 2000).

If spinal TRPV1 were sensitized after PAR2 activation, it is plausible that body temperature and/or endogenous ligands subsequently activated TRPV1. In addition it was demonstrated that activation of PAR2 reduced the temperature threshold required for TRPV1 activation to the body temperature in cultured cells (Dai et al., 2004). In our experiments intrathecal administration of staurosporine, a broad spectrum PKs inhibitor (with the highest affinity for PKC), partially attenuated the thermal hyperalgesia induced by spinal PAR2 activation in our experiments. This suggests the involvement of PKC in the process, most likely through phosphorylation of TRPV1 receptors (Numazaki et al., 2002, Amadesi et al., 2006, Chen et al., 2011).

6.1.2. Effect of PAR2 activation on nociceptive excitatory post-synaptic currents in superficial dorsal horn neurons

The potential underlying mechanisms of the behavioural changes were studied *in vitro.* In our experiments, the frequency of sEPSCs and amplitude of the dorsal root stimulation-evoked eEPSC were increased after PAR2 activating peptide (SLIGKV-NH$_2$) application. Similar increase of sEPSCs frequency induced with the same peptide (SLIGKV-NH$_2$) application was reported before in experiments with low concentration applications (3 µM and 5 µM; (Fujita et al., 2009). In contrast, bath application of other PAR2 activating peptide SLIGRL-NH$_2$ (10 µM) had no significant effect on the sEPSC frequency in lamina II neurons (Alier et al., 2008).

We newly demonstrated that application of PAR2 activating peptide increased the amplitude of evoked EPSCs and this effect was blocked by TRPV1 antagonist SB 366791, in addition the same mechanisms was present in PAR2-induced increase of sEPSCs frequency in our experiments. The sensitization of TRPV1 receptors by PAR2 activation was shown previously in DRG neurons (Amadesi et al., 2004). PAR2-induced effects on EPSCs in our recordings were mediated also by PKs in accordance with finding that PAR2 stimulation leads to TRPV1 sensitization via PKCε and PKA (Amadesi et al., 2006). Under our *in vitro* conditions with room temperature experiments it is more likely that endogenous substances may have activated spinal TRPV1 receptors. It was demonstrated before, that a low concentration of lipophilic endogenous ligand (N-oleoyldopamine, OLDA) activated sensitized TRPV1 receptors in spinal cord slices under similar conditions (Spicarova and Palecek, 2009). We cannot exclude the possibility that the dependence of TRPV1 activation on membrane voltage could also play a role in the

process (Matta and Ahern, 2007). In addition, PAR2 activation leads to enhanced release of pro-nociceptive peptides (SP, CGRP) from central endings of DRG neurons (Steinhoff et al., 2000, Amadesi et al., 2004) that may further modulate synaptic transmission and enhance nociceptive output from the spinal cord to the brain. The increase of sEPSC frequency by PAR2 activation could involve also mobilization of Ca^{2+} from intracellular stores and increased Ca^{2+} influx through other ion channels (Grant et al., 2007, Huang et al., 2012, Liu et al., 2013).

In the series of our experiments where TTX was present in the extracellular solution, PAR2 activation induced decrease of the mEPSCs frequency. Surprisingly, this decrease was also largely dependent on the TRPV1 receptor activation, while in other experiments TRPV1 receptors activation lead to increase of mEPSC frequency (Spicarova and Palecek, 2009, Spicarova et al., 2014a). These results indicate that under conditions, when TTX-sensitive sodium channels are blocked, another presynaptic mechanism induced by PAR2 activation predominated and resulted in decrease of glutamate release from the central endings of DRG neurons expressing also TRPV1 receptors. This observation could be explained by functional and physical connection between TRPV1 and large-conductance calcium- and voltage-activated potassium (BK) channels (Wu et al., 2013). On DRG neurons, TRPV1 and BK channels form complex, which could allow the activation of BK channels by increased local concentration of Ca^{2+} ions through TRPV1 (Wu et al., 2013). Due to outflow of K^+ ions from the cell through the BK channels, when TTX-sensitive Na^+ channels are blocked, the hyperpolarization could occur and the release of glutamate could be reduced. Another plausible mechanism could be the inhibition of voltage activated Ca^{2+} channels by TRPV1 activation. Olvanil, a non-pungent TRPV1 agonist, profoundly inhibited (approximately 60%) N-, P/Q-, L-, and R-type voltage-activated Ca^{2+} channel current in DRG neurons (Wu et al., 2006). The effect induced by olvanil was dependent on calmodulin and calcineurin activity. However, the mechanisms participating in TRPV1 activation and the subsequent intracellular responses may differ according to agonist used and receptor subtype (Liu et al., 1997). Recently, it was demonstrated that stochastic opening of voltage-activated Ca^{2+} channels is a major trigger for miniature glutamate release in hippocampal synapses (Ermolyuk et al., 2013). This finding supports the possible occurrence of decreased glutamate release from presynaptic endings of DRG neurons induced by PAR2 activation and mediated by TRPV1 modulation of voltage-activated Ca^{2+}

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channels in our conditions, when mEPSCs are recorded in acute spinal cord slices. Nevertheless, these two hypotheses require further investigation.

Miniature and spontaneous EPSCs both occur in spinal cord slices spontaneously without any stimulation. Fundamentally, mEPSCs are also spontaneous events, although during recording of mEPSC in our preparations potential self-generated formation and propagation of action potentials was avoided by blocking of sodium channels using TTX. It is suggested that mEPSCs reflect only the release of readily releasable pool of synaptic vesicles. In comparison evoked EPSCs were elicited by electrical stimulation of the dorsal root, the characteristic of current stimulus and the latency of eEPSC indicate the involvement of propagation of action potentials also in C-fibre. The PAR2-induced effect on sEPSCs and eEPSCs reflect more likely the mechanisms involved in physiological and pathophysiological conditions, whereas the opposite PAR2-induced effect on mEPSCs may be associated rather with experimental conditions.

Our results imply that PAR2 receptors may play an important role in nociceptive synaptic transmission at the spinal cord level, which is associated with hypersensitivity development. This PAR2-induced modulation of nociception is at least partially dependent on TRPV1 receptors activation. It seems plausible to suggest that their role may be potentiated during pathological processes, when both PAR2 and TRPV1 receptors expression is enhanced (Ji et al., 2002, Noorbakhsh et al., 2003, Bao et al., 2014b), moreover the increased TRPV1 expression in the superficial dorsal horn is dependent of PAR2 activation (Bao et al., 2015, Chen et al., 2015, Wei et al., 2016).

6.2. CCL2-induced modulation of synaptic transmission in the superficial spinal cord dorsal horn.

The contributions of the chemokine CCL2, its receptor CCR2 and TRPV1 receptors to nociceptive processing in the periphery are well documented, but their possible cooperation at the spinal cord level had not been studied.

Patch-clamp recordings showed the amplitude of the eEPSCs evoked by electrical stimulation of the dorsal root attached to a spinal cord slice was increased in 61% of the recorded neurons. This would suggest that CCL2-activated CCR2 receptors are present on the presynaptic endings of the central branches of DRG

neurons (White et al., 2005, Jung et al., 2008). Neurons in the superficial dorsal horn receive the majority of their synaptic input from nociceptive DRG neurons (Todd, 2010), which were shown to up-regulate CCR2 receptor expression under pathological conditions (Jung et al., 2008). During our recordings of eEPSCs, the activation of other spinal circuits could affect the observed results and thus diminish the effect of the CCL2-induced changes. When eEPSCs were recorded, most likely both myelinated and unmyelinated primary afferent fibers in the dorsal root were activated. The direct effect of CCL2 on the myelinated fibers was most likely limited as only a minority of the large diameter DRG neurons show CCR2 receptor expression (White et al., 2005, Jung et al., 2008). While this could be one of the reasons for the limited effect observed after CCL2 application in these experiments, the effect on the nociceptive primary afferents could still mediate behavioural changes. Recordings were made in population of superficial DH (laminae I–II) neurons, which presynaptic contacts with TRPV1/CCR2 receptors co-expressing primary afferents. Neurons without a response to the CCL2 application could belong to a subpopulation of DH neurons with presynaptic contacts expressing only TRPV1 receptors (as tested by capsaicin application). An inhibitory effect after CCL2 application was observed only in a few eEPSC recordings, where the activation of spinal circuits could affect the final result.

It was demonstrated previously that CCL2-induced hypersensitivity after intrathecal injection is mediated by CCR2 receptors (Dansereau et al., 2008). However, the existing evidence suggests a possible complex action of CCL2 in the spinal cord DH, especially under pathological conditions. CCL2 may be released from primary afferent fibers (Jung et al., 2008, Van Steenwinckel et al., 2011) and its expression is induced within minutes in spinal astrocytes (Gao et al., 2009b) while CCR2 receptors are localized predominantly in small and medium size DRG neurons (White et al., 2005, Jung et al., 2008), superficial and deep DH neurons (Gosselin et al., 2005, Gao et al., 2009b), astrocytes (Knerlich-Lukoschus et al., 2008) and presumably microglia (Abbadie et al., 2009, Old and Malcangio, 2012). An intrathecal injection of CCL2 activated spinal microglia in wild-type, but not in CCR2 knock-out mice (Zhang et al., 2007, Thacker et al., 2009) CCL2 application was also shown to induce the inhibition of voltage-dependent K^+ channels on DRG neurons (Sun et al., 2006) and to stimulate CGRP release from DRG neurons that was dependent on calcium influx via N-type voltage-activated Ca^{2+} channels,

calcium release from ryanodine-sensitive calcium stores and PLC and PKC activation (Qin et al., 2005). In our experiments, most likely CCR2 receptors on the presynaptic endings were activated after CCL2 application, leading to an increased release of glutamate from the synaptic terminals that were dependent on TRPV1 receptor activation.

6.2.1. Specific effect of CCL2 application on TRPV1 activation

Pre-treatment with a specific TRPV1 receptor antagonist (SB366791) reduced the effect of CCL2 on evoked EPSC currents recorded in superficial dorsal horn neurons. Cooperation between TRPV1 receptors and CCL2-activated CCR2 receptors was demonstrated previously in DRG neuronal cultures (Jung et al., 2008, Kao et al., 2012). CCL2-induced TRPV1 receptor sensitization was dependent on PKC and PLC activation, as was shown in HEK293 cells co-expressing CCR2 and TRPV1 receptors (Jung et al., 2008). CCL2 also induced nonselective cationic conductance in DRG neurons (Sun et al., 2006) that could be mediated by the nonselective cation channel of activated TRPV1 receptors (Oh et al., 1996). Phosphorylation of TRPV1 receptors by PKC or the activation of PLC could have an important role in the acute effects seen within minutes after CCL2 application in our experiments. Changes on the transcriptional level are less likely to be involved, as they require a longer time frame and also the bodies of DRG neurons were not present in our slice preparation. The fast effect of CCL2 application on the TRPV1 receptors expressing primary afferent fibers could be mediated by the activation of the G protein-coupled receptor CCR2, which in turn activates PLC to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP2). TRPV1 receptors are tonicaly inhibited by PIP2 and thus could become active following its hydrolysis (Chuang et al., 2001). To the TRPV1 receptor sensitization/activation by CCL2 can also contribute phosphorylation by PKC, which increases the TRPV1 channel open probability (Huang et al., 2006, Jung et al., 2008). Sensitized TRPV1 receptors, for instance by PKC phosphorylation, could be activated by endogenous agonists in the spinal cord (Spicarova and Palecek, 2009). The activation of TRPV1 receptors can lead to increased basal glutamate release (Ueda et al., 1993, Nakatsuka et al., 2002, Baccei et al., 2003), measured in previous experiments as an increase in spontaneous or miniature EPSC frequency (Spicarova and Palecek, 2009, 2010, Spicarova et al., 2011). Our results indicate that the acute application of CCL2 leads to an increase of

evoked glutamate release from the central endings of nociceptive primary afferent fibers and that this fast effect of CCL2 in the spinal cord is partially mediated by TRPV1 receptor activation.

The mechanisms by which CCL2 modulates the transmission of nociceptive signalling at the spinal cord level needs to be further investigated. However, our experiments suggest that spinal TRPV1 receptors may play an important role in this process that may underlie some of the neuropathic pain syndromes in patients.

6.3. Effect of paclitaxel on synaptic transmission at spinal cord level

This was the first time that functional interaction between TLR4 and TRPV1 has been shown in the spinal cord. My results showed that acute paclitaxel application induced significant increase of mESPC frequency that was absent in the presence of TRPV1 receptor antagonist SB366791. The results from our group then showed that TLR4 antagonist prevented paclitaxel evoked tachyphylaxis inhibition after second application of capsaicin. These findings suggest that paclitaxel activates TLR4 and signals downstream to modulate function of TRPV1 receptors. The downstream signalling pathways engaged by TLR4 to sensitize TRPV1 remain to be determined. Paclitaxel activated DRG neurons directly and increased the number of evoked action potentials and this was in the context of also altering the shape of the evoked action potentials (Li et al., 2015).

6.3.1. Specific signalling pathways between TLR4 and TRPV1

Possible explanation for the change of TRPV1 receptors responsiveness to capsaicin after paclitaxel application is activation this is hypothesizing engagement of the noncanonical TLR4 MAPK signal pathways. Previous experiments showed that paclitaxel treatment engages the signalling of ERK1/2 and p38 through TLR4. MAPK activation has also been shown to modulate the activities of ion channels such as sodium channel Nav1.7 and TRPV1 (Ji et al., 2002, Han et al., 2013), which have also been implicated as contributing to paclitaxel-related CIPN (Hara et al., 2013, Zhang and Dougherty, 2014). Another important consideration is that p38 and ERK1/2 integrate the activities of protein kinase signalling in DRG and spinal neurons (Hu and Gereau, 2003, Jin and Gereau, 2006, Han et al., 2012) and protein

kinase C is a well-defined pathway for regulation of TRPV1 signalling, including reversal of TRPV1 desensitization (Bhave et al., 2003, Han et al., 2012). PKC is also a component of TLR4 signalling that plays a key role in macrophage and dendritic cell activation in response to LPS (Aksoy et al., 2004). PKC is expressed in nociceptors and also has important roles in pain signalling (Numazaki et al., 2002, Vellani et al., 2004).

An important caveat to be considered is that, although an agonist effect of paclitaxel on TLR4 in rodents is widely accepted (Ding et al., 1990, Fitzpatrick and Wheeler, 2003, Javeed et al., 2009), such an effect for paclitaxel on human TLR4 is controversial. Some studies have reported pronounced cytokine production from human macrophages and other tissues by paclitaxel (Hashiguchi et al., 1982, White et al., 1998, Cassidy et al., 2002, Wang et al., 2002b), whereas others have not observed this (Manthey et al., 1993, Zimmer et al., 2008). TLR4 requires the accessory protein MD-2 for binding and activation by LPS and paclitaxel in murine macrophages (Fitzpatrick and Wheeler, 2003, Javeed et al., 2009). MD-2 is also required for activation of macrophages in humans by LPS, but it has been reported that paclitaxel binds MD-2 in a fashion that precludes activation of TLR4 (Kawasaki et al., 2001, Resman et al., 2008, Zimmer et al., 2008). It is difficult to reconcile this discrepancy, which has importance in the context here concerning the mechanisms of CIPN but, in a broader scope, concerning the utility of paclitaxel as a chemotherapeutic. A number of recent studies show a clear link between chemo-resistance and even the promotion of aggressiveness in many human cancer types by paclitaxel when TLR4 is expressed in these tissues(Szajnik et al., 2009, Geller et al., 2010, Volk-Draper et al., 2014). A possible explanation may be that an interaction between paclitaxel's binding to microtubules enhances its signalling via TLR4 (Fitzpatrick and Wheeler, 2003). This mechanism would clearly have a strong basis in DRG neurons and perhaps also those tumors expressing TLR4.

7. CONCLUSIONS

Modulation of synaptic transmission in the superficial dorsal horn neurons of spinal cord can significantly affect nociceptive signalling, especially in certain pathological pain states. Our results confirmed that spinal cord TRPV1 receptors play an important role in transmission of nociceptive signalling and that their modulation by different molecular mechanisms may be of crucial importance under pathological pain states. In this dissertation we have shown the following:

Activation of spinal PAR2 receptors by theirs activation peptide SLIGKV-NH$_2$ in behavioural experiments induced thermal hyperalgesia but did not affect responsiveness to mechanical stimuli. This thermal hyperalgesia could be significantly attenuated by intrathecal co-application of TRPV1 antagonist SB 366791 and reduced by PKC inhibitor staurosporine.

Application of PAR2 agonist induced inhibition of mEPSC frequency but led to increase of sEPSC frequency and increased amplitude of dorsal root stimulation evoked EPSC recorded in the dorsal horn neurons. These effects were also significantly attenuated by SB 366791 and staurosporine treatment. In control experiments PAR2 inactive peptide (VKGILS-NH$_2$) did not have any significant effect on the mEPSC, sEPSC frequency, evoked EPSC amplitude and animal behaviour.

Presynaptic PAR2 receptors may play an important role in modulation of nociceptive synaptic transmission in the spinal cord dorsal horn, especially under inflammatory conditions.

Our results also indicate that acute application of CCL2 leads to an increase of basal and evoked glutamate release from the central endings of nociceptive primary afferent fibers in the spinal cord recorded as an increase of eEPSC amplitude and that this effect is at least partially mediated by TRPV1 receptor activation.

Acute application of paclitaxel induced a significant increase of mEPSC frequency recorded in superficial dorsal horn neurons, that was dependent on TRPV1 receptors activation. This effect may play an important role in development of neuropathic pain states after chemotherapy treatments.

8. SUPPORT

9. LIST OF PUBLICATIONS

1) Spicarova D, Adamek P, Kalynovska N, **Mrozkova P**, Palecek J.

   TRPV1 receptor inhibition decreases CCL2-induced hyperalgesia.

   Neuropharmacology. Jun;81:75-84.doi: 2014

   (IF =5,106, Quartile 1, Times Cited: 8   2/2017)

2) Li Y, Adamek P, Zhang H, Tatsui CE, Rhines LD, **Mrozkova P**, Li Q, Kosturakis AK, Cassidy RM, Harrison DS, Cata JP, Sapire K, Zhang H, Kennamer-Chapman RM, Jawad AB, Ghetti A, Yan J, Palecek J, Dougherty PM.

   The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4.

   Journal of Neuroscience, Sep 30;35(39):13487-500. 2015

   (IF = 6.344, Quartile 1, Times Cited: 13   2/2017)

3) **Mrozkova P**, Palecek J, Spicarova D.

   The role of protease-activated receptor type 2 in nociceptive signalling and pain.

   Physiological Research, Jul 18;65(3):357-67. 2016.

   (IF = 1,643, Quartile 3, Times Cited:0   2/2017)

4) **Mrozkova P**, Spicarova D, Palecek J.

   Hypersensitivity Induced by Activation of Spinal Cord PAR2 Receptors Is Partially Mediated by TRPV1 Receptors.

   PLoS One, Oct 18;11(10):e0163991. 2016.

   (IF = 3.234, Quartile 1, Times Cited:0   2/2017)

10. REFERENCES

Abbadie C, Bhangoo S, De Koninck Y, Malcangio M, Melik-Parsadaniantz S, White FA (2009) Chemokines and pain mechanisms. Brain Res Rev 60:125-134.

Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ (2003) Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. Proceedings of the National Academy of Sciences of the United States of America 100:7947-7952.

Aksoy E, Goldman M, Willems F (2004) Protein kinase C epsilon: a new target to control inflammation and immune-mediated disorders. The international journal of biochemistry & cell biology 36:183-188.

Al-Ani B, Saifeddine M, Kawabata A, Renaux B, Mokashi S, Hollenberg MD (1999) Proteinase-activated receptor 2 (PAR(2)): development of a ligand-binding assay correlating with activation of PAR(2) by PAR(1)- and PAR(2)-derived peptide ligands. The Journal of pharmacology and experimental therapeutics 290:753-760.

Alier KA, Endicott JA, Stemkowski PL, Cenac N, Cellars L, Chapman K, Andrade-Gordon P, Vergnolle N, Smith PA (2008) Intrathecal administration of proteinase-activated receptor-2 agonists produces hyperalgesia by exciting the cell bodies of primary sensory neurons. The Journal of pharmacology and experimental therapeutics 324:224-233.

Almeida A, Lima D (1997) Activation by cutaneous or visceral noxious stimulation of spinal neurons projecting to the medullary dorsal reticular nucleus in the rat: a c-fos study. The European journal of neuroscience 9:686-695.

Antognini JF, Carstens EE, Raines DE (2002) Neural mechanisms of anesthesia. Totowa, N.J.: Humana ; Oxford : Blackwell.

Amadesi S, Cottrell GS, Divino L, Chapman K, Grady EF, Bautista F, Karanjia R, Barajas-Lopez C, Vanner S, Vergnolle N, Bunnett NW (2006) Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. The Journal of physiology 575:555-571.

Amadesi S, Grant AD, Cottrell GS, Vaksman N, Poole DP, Rozengurt E, Bunnett NW (2009) Protein kinase D isoforms are expressed in rat and mouse primary sensory neurons and are activated by agonists of protease-activated receptor 2. The Journal of comparative neurology 516:141-156.

Amadesi S, Nie J, Vergnolle N, Cottrell GS, Grady EF, Trevisani M, Manni C, Geppetti P, McRoberts JA, Ennes H, Davis JB, Mayer EA, Bunnett NW (2004) Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:4300-4312.

Angulo MC, Lambolez B, Audinat E, Hestrin S, Rossier J (1997) Subunit composition, kinetic, and permeation properties of AMPA receptors in single neocortical nonpyramidal cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:6685-6696.

Assas BM, Miyan JA, Pennock JL (2014) Cross-talk between neural and immune receptors provides a potential mechanism of homeostatic regulation in the gut mucosa. Mucosal immunology 7:1283-1289.

Baccei ML, Bardoni R, Fitzgerald M (2003) Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. The Journal of physiology 549:231-242.

Bao Y, Gao Y, Hou W, Yang L, Kong X, Zheng H, Li C, Hua B (2015) Engagement of signaling pathways of protease-activated receptor 2 and mu-opioid receptor in bone cancer pain and morphine tolerance. International journal of cancer Journal international du cancer 137:1475-1483.

Bao Y, Hou W, Hua B (2014a) Protease-activated receptor 2 signalling pathways: a role in pain processing. Expert opinion on therapeutic targets 18:15-27.

Bao Y, Hou W, Yang L, Liu R, Gao Y, Kong X, Shi Z, Li W, Zheng H, Jiang S, Hua B (2014b) Increased Expression of Protease-Activated Receptor 2 and 4 Within Dorsal Root Ganglia in a Rat Model of Bone Cancer Pain. Journal of molecular neuroscience : MN.

Barry MF, Ziff EB (2002) Receptor trafficking and the plasticity of excitatory synapses. Current opinion in neurobiology 12:279-286.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267-284.

Benarroch EE (2008) TRP channels: functions and involvement in neurologic disease. Neurology 70:648-652.

Benham CD, Gunthorpe MJ, Davis JB (2003) TRPV channels as temperature sensors. Cell Calcium 33:479-487.

Bespalov AY, Zvartau EE, Beardsley PM (2001) Opioid-NMDA receptor interactions may clarify conditioned (associative) components of opioid analgesic tolerance. Neuroscience and biobehavioral reviews 25:343-353.

Bhangoo S, Ren D, Miller RJ, Henry KJ, Lineswala J, Hamdouchi C, Li B, Monahan PE, Chan DM, Ripsch MS, White FA (2007) Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. Mol Pain 3:38.

Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proceedings of the National Academy of Sciences of the United States of America 100:12480-12485.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. The Journal of physiology 232:331-356.

Boehm J, Malinow R (2005) AMPA receptor phosphorylation during synaptic plasticity. Biochemical Society transactions 33:1354-1356.

Bohm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG, Bunnett NW (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. The Biochemical journal 314 (Pt 3):1009-1016.

Bonnington JK, McNaughton PA (2003) Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor. The Journal of physiology 551:433-446.

Boyette-Davis J, Dougherty PM (2011) Protection against oxaliplatin-induced mechanical hyperalgesia and intraepidermal nerve fiber loss by minocycline. Exp Neurol 229:353-357.

Boyette-Davis JA, Cata JP, Driver LC, Novy DM, Bruel BM, Mooring DL, Wendelschafer-Crabb G, Kennedy WR, Dougherty PM (2013) Persistent chemoneuropathy in patients receiving the plant alkaloids paclitaxel and vincristine. Cancer chemotherapy and pharmacology 71:619-626.

Boyette-Davis JA, Cata JP, Zhang H, Driver LC, Wendelschafer-Crabb G, Kennedy WR, Dougherty PM (2011) Follow-up psychophysical studies in bortezomib-related chemoneuropathy patients. J Pain 12:1017-1024.

Brass LF, Molino M (1997) Protease-activated G protein-coupled receptors on human platelets and endothelial cells. Thrombosis and haemostasis 78:234-241.

Bredesen DE (2009) Neurodegeneration in Alzheimer's disease: caspases and synaptic element interdependence. Molecular neurodegeneration 4:27.

Breivik H, Borchgrevink PC, Allen SM, Rosseland LA, Romundstad L, Hals EK, Kvarstein G, Stubhaug A (2008) Assessment of pain. British journal of anaesthesia 101:17-24.

Bsibsi M, Ravid R, Gveric D, van Noort JM (2002) Broad expression of Toll-like receptors in the human central nervous system. Journal of neuropathology and experimental neurology 61:1013-1021.

Burgess PR, Perl ER (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. The Journal of physiology 190:541-562.

Camerer E, Huang W, Coughlin SR (2000) Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proceedings of the National Academy of Sciences of the United States of America 97:5255-5260.

Carlson JD, Maire JJ, Martenson ME, Heinricher MM (2007) Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:13222-13231.

Carlton SM, Coggeshall RE (2001) Peripheral capsaicin receptors increase in the inflamed rat hindpaw: a possible mechanism for peripheral sensitization. Neuroscience letters 310:53-56.

Cassidy PB, Moos PJ, Kelly RC, Fitzpatrick FA (2002) Cyclooxygenase-2 induction by paclitaxel, docetaxel, and taxane analogues in human monocytes and murine macrophages: structure-activity relationships and their implications. Clinical cancer research : an official journal of the American Association for Cancer Research 8:846-855.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306-313.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.

Cavanaugh DJ, Chesler AT, Braz JM, Shah NM, Julius D, Basbaum AI (2011b) Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:10119-10127.

Cenac N (2013) Protease-activated receptors as therapeutic targets in visceral pain. Current neuropharmacology 11:598-605.

Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, Wallace JL, Hollenberg MD, Bunnett NW, Garcia-Villar R, Bueno L, Vergnolle N (2002) Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. The American journal of pathology 161:1903-1915.

Cenac N, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, Coelho AM, Fioramonti J, Bueno L (2003) Proteinase-activated receptor-2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability. J Immunol 170:4296-4300.

Cenac N, Vergnolle N (2005) Proteases and protease-activated receptors (PARs): novel signals for pain. Curr Top Med Chem 5:569-576.

Cervero F, Laird JM (1999) Visceral pain. Lancet 353:2145-2148.

Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999) Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. Neuron 23:617-624.

Cesare P, McNaughton P (1996) A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. Proceedings of the National Academy of Sciences of the United States of America 93:15435-15439.

Clark AK, Staniland AA, Malcangio M (2011) Fractalkine/CX3CR1 signalling in chronic pain and inflammation. Curr Pharm Biotechnol 12:1707-1714.

Clark AK, Yip PK, Grist J, Gentry C, Staniland AA, Marchand F, Dehvari M, Wotherspoon G, Winter J, Ullah J, Bevan S, Malcangio M (2007) Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proceedings of the National Academy of Sciences of the United States of America 104:10655-10660.

Cortright DN, Szallasi A (2004) Biochemical pharmacology of the vanilloid receptor TRPV1. An update. European journal of biochemistry / FEBS 271:1814-1819.

Corvera CU, Dery O, McConalogue K, Bohm SK, Khitin LM, Caughey GH, Payan DG, Bunnett NW (1997) Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. The Journal of clinical investigation 100:1383-1393.

Corvera CU, Dery O, McConalogue K, Gamp P, Thoma M, Al-Ani B, Caughey GH, Hollenberg MD, Bunnett NW (1999) Thrombin and mast cell tryptase regulate guinea-pig myenteric neurons through proteinase-activated receptors-1 and -2. The Journal of physiology 517 (Pt 3):741-756.

D'Andrea MR, Derian CK, Leturcq D, Baker SM, Brunmark A, Ling P, Darrow AL, Santulli RJ, Brass LF, Andrade-Gordon P (1998) Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 46:157-164.

Dai Y, Moriyama T, Higashi T, Togashi K, Kobayashi K, Yamanaka H, Tominaga M, Noguchi K (2004) Proteinase-activated receptor 2-mediated potentiation of transient receptor potential vanilloid subfamily 1 activity reveals a mechanism for proteinase-induced inflammatory

pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:4293-4299.

Dai Y, Wang S, Tominaga M, Yamamoto S, Fukuoka T, Higashi T, Kobayashi K, Obata K, Yamanaka H, Noguchi K (2007) Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. The Journal of clinical investigation 117:1979-1987.

Dansereau MA, Gosselin RD, Pohl M, Pommier B, Mechighel P, Mauborgne A, Rostene W, Kitabgi P, Beaudet N, Sarret P, Melik-Parsadaniantz S (2008) Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. Journal of neurochemistry 106:757-769.

de Garavilla L, Vergnolle N, Young SH, Ennes H, Steinhoff M, Ossovskaya VS, D'Andrea MR, Mayer EA, Wallace JL, Hollenberg MD, Andrade-Gordon P, Bunnett NW (2001) Agonists of proteinase-activated receptor 1 induce plasma extravasation by a neurogenic mechanism. British journal of pharmacology 133:975-987.

DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. The Journal of cell biology 148:1267-1281.

Dery O, Corvera CU, Steinhoff M, Bunnett NW (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. The American journal of physiology 274:C1429-1452.

Ding AH, Porteu F, Sanchez E, Nathan CF (1990) Shared actions of endotoxin and taxol on TNF receptors and TNF release. Science 248:370-372.

Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM (2011) LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. Journal of dental research 90:759-764.

Docherty RJ, Yeats JC, Bevan S, Boddeke HW (1996) Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. Pflugers Archiv : European journal of physiology 431:828-837.

Ermolyuk YS, Alder FG, Surges R, Pavlov IY, Timofeeva Y, Kullmann DM, Volynski KE (2013) Differential triggering of spontaneous glutamate release by P/Q-, N- and R-type Ca2+ channels. Nature neuroscience 16:1754-1763.

Fan Y, Chen J, Ye J, Yan H, Cai Y (2014) Proteinase-activated receptor 2 modulates corticotropin releasing hormone-induced brain-derived neurotrophic factor release from microglial cells. Cell biology international 38:92-96.

Fernandez-Carvajal A, Fernandez-Ballester G, Devesa I, Gonzalez-Ros JM, Ferrer-Montiel A (2011) New strategies to develop novel pain therapies: addressing thermoreceptors from different points of view. Pharmaceuticals (Basel) 5:16-48.

Fernandez EJ, Lolis E (2002) Structure, function, and inhibition of chemokines. Annual review of pharmacology and toxicology 42:469-499.

Ferreira J, da Silva GL, Calixto JB (2004) Contribution of vanilloid receptors to the overt nociception induced by B2 kinin receptor activation in mice. British journal of pharmacology 141:787-794.

Ferrini F, Salio C, Vergnano AM, Merighi A (2007) Vanilloid receptor-1 (TRPV1)-dependent activation of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse. Pain 129:195-209.

Fiorucci S, Distrutti E (2002) Role of PAR2 in pain and inflammation. Trends in pharmacological sciences 23:153-155.

Fitzpatrick FA, Wheeler R (2003) The immunopharmacology of paclitaxel (Taxol), docetaxel (Taxotere), and related agents. International immunopharmacology 3:1699-1714.

Froemke RC, Dan Y (2002) Spike-timing-dependent synaptic modification induced by natural spike trains. Nature 416:433-438.

Fu ES, Zhang YP, Sagen J, Candiotti KA, Morton PD, Liebl DJ, Bethea JR, Brambilla R (2010) Transgenic inhibition of glial NF-kappa B reduces pain behavior and inflammation after peripheral nerve injury. Pain 148:509-518.

Fujita T, Liu T, Nakatsuka T, Kumamoto E (2009) Proteinase-activated receptor-1 activation presynaptically enhances spontaneous glutamatergic excitatory transmission in adult rat substantia gelatinosa neurons. J Neurophysiol 102:312-319.

Gao YJ, Cheng JK, Zeng Q, Xu ZZ, Decosterd I, Xu X, Ji RR (2009a) Selective inhibition of JNK with a peptide inhibitor attenuates pain hypersensitivity and tumor growth in a mouse skin cancer pain model. Exp Neurol 219:146-155.

Gao YJ, Zhang L, Samad OA, Suter MR, Yasuhiko K, Xu ZZ, Park JY, Lind AL, Ma Q, Ji RR (2009b) JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:4096-4108.

Garcia-Sanz N, Fernandez-Carvajal A, Morenilla-Palao C, Planells-Cases R, Fajardo-Sanchez E, Fernandez-Ballester G, Ferrer-Montiel A (2004) Identification of a tetramerization domain in the C terminus of the vanilloid receptor. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:5307-5314.

Garland EL (2012) Pain processing in the human nervous system: a selective review of nociceptive and biobehavioral pathways. Primary care 39:561-571.

Gelinas C, Loiselle CG, LeMay S, Ranger M, Bouchard E, McCormack D (2008) Theoretical, psychometric, and pragmatic issues in pain measurement. Pain management nursing : official journal of the American Society of Pain Management Nurses 9:120-130.

Geller MA, Bui-Nguyen TM, Rogers LM, Ramakrishnan S (2010) Chemotherapy induces macrophage chemoattractant protein-1 production in ovarian cancer. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society 20:918-925.

Goodsell, D.S., 2011. Toll-like Receptors. *RCSB Protein Data Bank* [online]. Dostupné z: doi:10.2210/rcsb_pdb/mom_2011_11

Gosselin RD, Varela C, Banisadr G, Mechighel P, Rostene W, Kitabgi P, Melik-Parsadaniantz S (2005) Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. Journal of neurochemistry 95:1023-1034.

Grace PM, Hutchinson MR, Maier SF, Watkins LR (2014) Pathological pain and the neuroimmune interface. Nature reviews Immunology 14:217-231.

Grant AD, Cottrell GS, Amadesi S, Trevisani M, Nicoletti P, Materazzi S, Altier C, Cenac N, Zamponi GW, Bautista-Cruz F, Lopez CB, Joseph EK, Levine JD, Liedtke W, Vanner S, Vergnolle N, Geppetti P, Bunnett NW (2007) Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. The Journal of physiology 578:715-733.

Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M (2002) Heat-evoked activation of the ion channel, TRPV4. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:6408-6414.

Gunthorpe MJ, Chizh BA (2009) Clinical development of TRPV1 antagonists: targeting a pivotal point in the pain pathway. Drug discovery today 14:56-67.

Gunthorpe MJ, Rami HK, Jerman JC, Smart D, Gill CH, Soffin EM, Luis Hannan S, Lappin SC, Egerton J, Smith GD, Worby A, Howett L, Owen D, Nasir S, Davies CH, Thompson M, Wyman PA, Randall AD, Davis JB (2004) Identification and characterisation of SB-366791, a potent and selective vanilloid receptor (VR1/TRPV1) antagonist. Neuropharmacology 46:133-149.

Guo A, Vulchanova L, Wang J, Li X, Elde R (1999) Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. The European journal of neuroscience 11:946-958.

Hagiwara H, Sunada Y (2004) Mechanism of taxane neurotoxicity. Breast Cancer 11:82-85.

Han J, Lee JD, Bibbs L, Ulevitch RJ (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265:808-811.

Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, Kim Y, Nie H, Qu L, Patel KN, Li Z, McNeil B, He S, Guan Y, Xiao B, Lamotte RH, Dong X (2013) A subpopulation of nociceptors specifically linked to itch. Nature neuroscience 16:174-182.

Han Y, Li Y, Xiao X, Liu J, Meng XL, Liu FY, Xing GG, Wan Y (2012) Formaldehyde up-regulates TRPV1 through MAPK and PI3K signaling pathways in a rat model of bone cancer pain. Neuroscience bulletin 28:165-172.

Hara T, Chiba T, Abe K, Makabe A, Ikeno S, Kawakami K, Utsunomiya I, Hama T, Taguchi K (2013) Effect of paclitaxel on transient receptor potential vanilloid 1 in rat dorsal root ganglion. Pain 154:882-889.

Hashiguchi T, Kobayashi H, Tosaka T, Libet B (1982) Two muscarinic depolarizing mechanisms in mammalian sympathetic neurons. Brain research 242:378-382.

Hayes P, Meadows HJ, Gunthorpe MJ, Harries MH, Duckworth DM, Cairns W, Harrison DC, Clarke CE, Ellington K, Prinjha RK, Barton AJ, Medhurst AD, Smith GD, Topp S, Murdock P, Sanger GJ, Terrett J, Jenkins O, Benham CD, Randall AD, Gloger IS, Davis JB (2000) Cloning and functional expression of a human orthologue of rat vanilloid receptor-1. Pain 88:205-215.

Heinricher MM, Tavares I, Leith JL, Lumb BM (2009) Descending control of nociception: Specificity, recruitment and plasticity. Brain Res Rev 60:214-225.

Hewitt E, Pitcher T, Rizoska B, Tunblad K, Henderson I, Sahlberg BL, Grabowska U, Classon B, Edenius C, Malcangio M, Lindstrom E (2016) Selective Cathepsin S Inhibition with MIV-247 Attenuates Mechanical Allodynia and Enhances the Antiallodynic Effects of Gabapentin and Pregabalin in a Mouse Model of Neuropathic Pain. The Journal of pharmacology and experimental therapeutics 358:387-396.

Hill K, Schaefer M (2007) TRPA1 is differentially modulated by the amphipathic molecules trinitrophenol and chlorpromazine. The Journal of biological chemistry 282:7145-7153.

Hollenberg MD, Compton SJ (2002) International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacological reviews 54:203-217.

Hollenberg MD, Saifeddine M, al-Ani B, Kawabata A (1997) Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. Canadian journal of physiology and pharmacology 75:832-841.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annual review of neuroscience 17:31-108.

Hoogerwerf WA, Shenoy M, Winston JH, Xiao SY, He Z, Pasricha PJ (2004) Trypsin mediates nociception via the proteinase-activated receptor 2: a potentially novel role in pancreatic pain. Gastroenterology 127:883-891.

Hoogerwerf WA, Zou L, Shenoy M, Sun D, Micci MA, Lee-Hellmich H, Xiao SY, Winston JH, Pasricha PJ (2001) The proteinase-activated receptor 2 is involved in nociception. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:9036-9042.

Hu HJ, Gereau RWt (2003) ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. II. Modulation of neuronal excitability. J Neurophysiol 90:1680-1688.

Huang J, Zhang X, McNaughton PA (2006) Modulation of temperature-sensitive TRP channels. Seminars in cell & developmental biology 17:638-645.

Huang SM, Bisogno T, Trevisani M, Al-Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros TJ, Krey JF, Chu CJ, Miller JD, Davies SN, Geppetti P, Walker JM, Di Marzo V (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. Proceedings of the National Academy of Sciences of the United States of America 99:8400-8405.

Huang Z, Tao K, Zhu H, Miao X, Wang Z, Yu W, Lu Z (2011) Acute PAR2 activation reduces GABAergic inhibition in the spinal dorsal horn. Brain research 1425:20-26.

Huang ZJ, Li HC, Cowan AA, Liu S, Zhang YK, Song XJ (2012) Chronic compression or acute dissociation of dorsal root ganglion induces cAMP-dependent neuronal hyperexcitability through activation of PAR2. Pain 153:1426-1437.

Hunt SP, Rossi J (1985) Peptide- and non-peptide-containing unmyelinated primary afferents: the parallel processing of nociceptive information. Philos Trans R Soc Lond B Biol Sci 308:283-289.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D, Oh U (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proceedings of the National Academy of Sciences of the United States of America 97:6155-6160.

Chacur M, Lambertz D, Hoheisel U, Mense S (2009) Role of spinal microglia in myositis-induced central sensitisation: an immunohistochemical and behavioural study in rats. Eur J Pain 13:915-923.

Chang YW, Waxman SG (2010) Minocycline attenuates mechanical allodynia and central sensitization following peripheral second-degree burn injury. J Pain 11:1146-1154.

Chaudhry V, Rowinsky EK, Sartorius SE, Donehower RC, Cornblath DR (1994) Peripheral neuropathy from taxol and cisplatin combination chemotherapy: clinical and electrophysiological studies. Annals of neurology 35:304-311.

Chen K, Zhang ZF, Liao MF, Yao WL, Wang J, Wang XR (2015) Blocking PAR2 attenuates oxaliplatin-induced neuropathic pain via TRPV1 and releases of substance P and CGRP in superficial dorsal horn of spinal cord. Journal of the neurological sciences 352:62-67.

Chen Y, Willcockson HH, Valtschanoff JG (2009) Influence of the vanilloid receptor TRPV1 on the activation of spinal cord glia in mouse models of pain. Exp Neurol 220:383-390.

Chen Y, Yang C, Wang ZJ (2011) Proteinase-activated receptor 2 sensitizes transient receptor potential vanilloid 1, transient receptor potential vanilloid 4, and transient receptor potential ankyrin 1 in paclitaxel-induced neuropathic pain. Neuroscience 193:440-451.

Chiang CY, Li Z, Dostrovsky JO, Sessle BJ (2010) Central sensitization in medullary dorsal horn involves gap junctions and hemichannels. Neuroreport 21:233-237.

Christianson CA, Dumlao DS, Stokes JA, Dennis EA, Svensson CI, Corr M, Yaksh TL (2011) Spinal TLR4 mediates the transition to a persistent mechanical hypersensitivity after the resolution of inflammation in serum-transferred arthritis. Pain 152:2881-2891.

Chu CJ, Huang SM, De Petrocellis L, Bisogno T, Ewing SA, Miller JD, Zipkin RE, Daddario N, Appendino G, Di Marzo V, Walker JM (2003) N-oleoyldopamine, a novel endogenous capsaicin-like lipid that produces hyperalgesia. The Journal of biological chemistry 278:13633-13639.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411:957-962.

Jancso-Gabor A, Szolcsanyi J, Jancso N (1970) Irreversible impairment of thermoregulation induced by capsaicin and similar pungent substances in rats and guinea-pigs. The Journal of physiology 206:495-507.

Javeed A, Ashraf M, Riaz A, Ghafoor A, Afzal S, Mukhtar MM (2009) Paclitaxel and immune system. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 38:283-290.

Ji RR, Gereau RWt, Malcangio M, Strichartz GR (2009) MAP kinase and pain. Brain Res Rev 60:135-148.

Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36:57-68.

Ji RR, Strichartz G (2004) Cell signaling and the genesis of neuropathic pain. Science's STKE : signal transduction knowledge environment 2004:reE14.

Jin X, Gereau RWt (2006) Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-alpha. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:246-255.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.

Jung H, Toth PT, White FA, Miller RJ (2008) Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. Journal of neurochemistry 104:254-263.

Kandel ER, Schwartz JH, Jessell TM (2000) Principles of neural science, 4th ed.Edition. New York ; London: McGraw-Hill, Health Professions Division.

Kanke T, Macfarlane SR, Seatter MJ, Davenport E, Paul A, McKenzie RC, Plevin R (2001) Proteinase-activated receptor-2-mediated activation of stress-activated protein kinases and inhibitory kappa B kinases in NCTC 2544 keratinocytes. The Journal of biological chemistry 276:31657-31666.

Kao DJ, Li AH, Chen JC, Luo RS, Chen YL, Lu JC, Wang HL (2012) CC chemokine ligand 2 upregulates the current density and expression of TRPV1 channels and Nav1.8 sodium channels in dorsal root ganglion neurons. J Neuroinflammation 9:189.

Kawabata A (2011) Prostaglandin E2 and pain--an update. Biol Pharm Bull 34:1170-1173.

Kawabata A, Kanke T, Yonezawa D, Ishiki T, Saka M, Kabeya M, Sekiguchi F, Kubo S, Kuroda R, Iwaki M, Katsura K, Plevin R (2004) Potent and metabolically stable agonists for protease-activated receptor-2: evaluation of activity in multiple assay systems in vitro and in vivo. The Journal of pharmacology and experimental therapeutics 309:1098-1107.

Kawabata A, Saifeddine M, Al-Ani B, Leblond L, Hollenberg MD (1999) Evaluation of proteinase-activated receptor-1 (PAR1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR2 by PAR1-targeted ligands. The Journal of pharmacology and experimental therapeutics 288:358-370.

Kawasaki K, Gomi K, Nishijima M (2001) Cutting edge: Gln22 of mouse MD-2 is essential for species-specific lipopolysaccharide mimetic action of taxol. J Immunol 166:11-14.

Kim DY, Kim SH, Choi HB, Min C, Gwag BJ (2001) High abundance of GluR1 mRNA and reduced Q/R editing of GluR2 mRNA in individual NADPH-diaphorase neurons. Molecular and cellular neurosciences 17:1025-1033.

Kim YH, Park CK, Back SK, Lee CJ, Hwang SJ, Bae YC, Na HS, Kim JS, Jung SJ, Oh SB (2009b) Membrane-delimited coupling of TRPV1 and mGluR5 on presynaptic terminals of nociceptive neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:10000-10009.

Knerlich-Lukoschus F, Juraschek M, Blomer U, Lucius R, Mehdorn HM, Held-Feindt J (2008) Force-dependent development of neuropathic central pain and time-related CCL2/CCR2 expression after graded spinal cord contusion injuries of the rat. Journal of neurotrauma 25:427-448.

Koetzner L, Gregory JA, Yaksh TL (2004) Intrathecal protease-activated receptor stimulation produces thermal hyperalgesia through spinal cyclooxygenase activity. The Journal of pharmacology and experimental therapeutics 311:356-363.

Koltzenburg M, Lundberg LE, Torebjork HE (1992) Dynamic and static components of mechanical hyperalgesia in human hairy skin. Pain 51:207-219.

Koplas PA, Rosenberg RL, Oxford GS (1997) The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:3525-3537.

Kosturakis AK, He Z, Li Y, Boyette-Davis JA, Shah N, Thomas SK, Zhang H, Vichaya EG, Wang XS, Wendelschafer-Crabb G, Kennedy WR, Simone DA, Cleeland CS, Dougherty PM (2014) Subclinical peripheral neuropathy in patients with multiple myeloma before chemotherapy is correlated with decreased fingertip innervation density. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 32:3156-3162.

Lakhan SE, Caro M, Hadzimichalis N (2013) NMDA Receptor Activity in Neuropsychiatric Disorders. Frontiers in psychiatry 4:52.

Lam DK, Dang D, Zhang J, Dolan JC, Schmidt BL (2012) Novel animal models of acute and chronic cancer pain: a pivotal role for PAR2. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:14178-14183.

Lam DK, Schmidt BL (2010) Serine proteases and protease-activated receptor 2-dependent allodynia: a novel cancer pain pathway. Pain 149:263-272.

LaMotte RH, Thalhammer JG, Torebjork HE, Robinson CJ (1982) Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat. The Journal of neuroscience : the official journal of the Society for Neuroscience 2:765-781.

Lappin SC, Randall AD, Gunthorpe MJ, Morisset V (2006) TRPV1 antagonist, SB-366791, inhibits glutamatergic synaptic transmission in rat spinal dorsal horn following peripheral inflammation. European journal of pharmacology 540:73-81.

Laursen WJ, Bagriantsev SN, Gracheva EO (2014) TRPA1 channels: chemical and temperature sensitivity. Current topics in membranes 74:89-112.

Lester RA, Clements JD, Westbrook GL, Jahr CE (1990) Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. Nature 346:565-567.

Leszkiewicz DN, Kandler K, Aizenman E (2000) Enhancement of NMDA receptor-mediated currents by light in rat neurones in vitro. The Journal of physiology 524 Pt 2:365-374.

Li D, Fu Y, Zhang W, Su G, Liu B, Guo M, Li F, Liang D, Liu Z, Zhang X, Cao Y, Zhang N, Yang Z (2013) Salidroside attenuates inflammatory responses by suppressing nuclear factor-kappaB and mitogen activated protein kinases activation in lipopolysaccharide-induced mastitis in mice. Inflammation research : official journal of the European Histamine Research Society [et al] 62:9-15.

Li Y, Adamek P, Zhang H, Tatsui CE, Rhines LD, Mrozkova P, Li Q, Kosturakis AK, Cassidy RM, Harrison DS, Cata JP, Sapire K, Kennamer-Chapman RM, Jawad AB, Ghetti A, Yan J, Palecek J, Dougherty PM (2015) The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4. The Journal of neuroscience : the official journal of the Society for Neuroscience 35:13487-13500.

Li Y, Zhang H, Kosturakis AK, Jawad AB, Dougherty PM (2014) Toll-like receptor 4 signaling contributes to Paclitaxel-induced peripheral neuropathy. J Pain 15:712-725.

Liao M, Cao E, Julius D, Cheng Y (2013) Structure of the TRPV1 ion channel determined by electron cryo-microscopy. Nature 504:107-112.

Lilienbaum A, Israel A (2003) From calcium to NF-kappa B signaling pathways in neurons. Molecular and cellular biology 23:2680-2698.

Linden DR, Manning BP, Bunnett NW, Mawe GM (2001) Agonists of proteinase-activated receptor 2 excite guinea pig ileal myenteric neurons. European journal of pharmacology 431:311-314.

Lishko PV, Procko E, Jin X, Phelps CB, Gaudet R (2007) The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. Neuron 54:905-918.

Liu L, Lo Y, Chen I, Simon SA (1997) The responses of rat trigeminal ganglion neurons to capsaicin and two nonpungent vanilloid receptor agonists, olvanil and glyceryl nonamide. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:4101-4111.

Liu S, Liu YP, Yue DM, Liu GJ (2013) Protease-activated receptor 2 in dorsal root ganglion contributes to peripheral sensitization of bone cancer pain. Eur J Pain.

Liu T, Berta T, Xu ZZ, Park CK, Zhang L, Lu N, Liu Q, Liu Y, Gao YJ, Liu YC, Ma Q, Dong X, Ji RR (2012) TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. The Journal of clinical investigation 122:2195-2207.

Liu T, Xu ZZ, Park CK, Berta T, Ji RR (2010) Toll-like receptor 7 mediates pruritus. Nature neuroscience 13:1460-1462.

Loeser JD, Treede RD (2008) The Kyoto protocol of IASP Basic Pain Terminology. Pain 137:473-477.

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R (2001) Proteinase-activated receptors. Pharmacological reviews 53:245-282.

Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF, Patapoutian A (2007) Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. Nature 445:541-545.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Manthey CL, Qureshi N, Stutz PL, Vogel SN (1993) Lipopolysaccharide antagonists block taxol-induced signaling in murine macrophages. The Journal of experimental medicine 178:695-702.

Marchettini P, Simone DA, Caputi G, Ochoa JL (1996) Pain from excitation of identified muscle nociceptors in humans. Brain research 740:109-116.

Matta JA, Ahern GP (2007) Voltage is a partial activator of rat thermosensitive TRP channels. The Journal of physiology 585:469-482.

Matthews CC, Zielke HR, Parks DA, Fishman PS (2003) Glutamate-pyruvate transaminase protects against glutamate toxicity in hippocampal slices. Brain research 978:59-64.

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416:52-58.

Mehta MR (2004) Cooperative LTP can map memory sequences on dendritic branches. Trends Neurosci 27:69-72.

Merskey H, Bogduk N, eds. (1994) Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain terms prepared by the International Association for the Study of Pain,Task Force of Taxonomy. In. Seattle: IASP Press.

Millan MJ (1999) The induction of pain: an integrative review. Progress in neurobiology 57:1-164.

Moiseenkova-Bell VY, Stanciu LA, Serysheva, II, Tobe BJ, Wensel TG (2008) Structure of TRPV1 channel revealed by electron cryomicroscopy. Proceedings of the National Academy of Sciences of the United States of America 105:7451-7455.

Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, Hoxie JA, Schechter N, Woolkalis M, Brass LF (1997) Interactions of mast cell tryptase with thrombin receptors and PAR-2. The Journal of biological chemistry 272:4043-4049.

Morenilla-Palao C, Planells-Cases R, Garcia-Sanz N, Ferrer-Montiel A (2004) Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. The Journal of biological chemistry 279:25665-25672.

Mule F, Baffi MC, Falzone M, Cerra MC (2002) Signal transduction pathways involved in the mechanical responses to protease-activated receptors in rat colon. The Journal of pharmacology and experimental therapeutics 303:1265-1272.

Nagy I, Friston D, Valente JS, Torres Perez JV, Andreou AP (2014) Pharmacology of the capsaicin receptor, transient receptor potential vanilloid type-1 ion channel. Progress in drug

research Fortschritte der Arzneimittelforschung Progres des recherches pharmaceutiques 68:39-76.

Nakatsuka T, Furue H, Yoshimura M, Gu JG (2002) Activation of central terminal vanilloid receptor-1 receptors and alpha beta-methylene-ATP-sensitive P2X receptors reveals a converged synaptic activity onto the deep dorsal horn neurons of the spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:1228-1237.

Namer B, Reeh P (2013) Scratching an itch. Nature neuroscience 16:117-118.

Narumiya S, Sugimoto Y, Ushikubi F (1999) Prostanoid receptors: structures, properties, and functions. Physiological reviews 79:1193-1226.

Noorbakhsh F, Tsutsui S, Vergnolle N, Boven LA, Shariat N, Vodjgani M, Warren KG, Andrade-Gordon P, Hollenberg MD, Power C (2006) Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. The Journal of experimental medicine 203:425-435.

Noorbakhsh F, Vergnolle N, Hollenberg MD, Power C (2003) Proteinase-activated receptors in the nervous system. Nature reviews Neuroscience 4:981-990.

Numazaki M, Tominaga T, Toyooka H, Tominaga M (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. The Journal of biological chemistry 277:13375-13378.

Nystedt S, Emilsson K, Wahlestedt C, Sundelin J (1994) Molecular cloning of a potential proteinase activated receptor. Proceedings of the National Academy of Sciences of the United States of America 91:9208-9212.

Nystedt S, Larsson AK, Aberg H, Sundelin J (1995) The mouse proteinase-activated receptor-2 cDNA and gene. Molecular cloning and functional expression. The Journal of biological chemistry 270:5950-5955.

Nystedt S, Ramakrishnan V, Sundelin J (1996) The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. The Journal of biological chemistry 271:14910-14915.

O'Brien JM, Jr., Wewers MD, Moore SA, Allen JN (1995) Taxol and colchicine increase LPS-induced pro-IL-1 beta production, but do not increase IL-1 beta secretion. A role for microtubules in the regulation of IL-1 beta production. J Immunol 154:4113-4122.

Oh U, Hwang SW, Kim D (1996) Capsaicin activates a nonselective cation channel in cultured neonatal rat dorsal root ganglion neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 16:1659-1667.

Old EA, Malcangio M (2012) Chemokine mediated neuron-glia communication and aberrant signalling in neuropathic pain states. Curr Opin Pharmacol 12:67-73.

Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors: contribution to physiology and disease. Physiological reviews 84:579-621.

Patwardhan AM, Scotland PE, Akopian AN, Hargreaves KM (2009) Activation of TRPV1 in the spinal cord by oxidized linoleic acid metabolites contributes to inflammatory hyperalgesia. Proceedings of the National Academy of Sciences of the United States of America 106:18820-18824.

Paulsen O, Sejnowski TJ (2000) Natural patterns of activity and long-term synaptic plasticity. Current opinion in neurobiology 10:172-179.

Peng C, Aron L, Klein R, Li M, Wurst W, Prakash N, Le W (2011) Pitx3 is a critical mediator of GDNF-induced BDNF expression in nigrostriatal dopaminergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:12802-12815.

Perl ER, Kumazawa T, Lynn B, Kenins P (1976) Sensitization of high threshold receptors with unmyelinated (C) afferent fibers. Progress in brain research 43:263-277.

Poole DP, Amadesi S, Veldhuis NA, Abogadie FC, Lieu T, Darby W, Liedtke W, Lew MJ, McIntyre P, Bunnett NW (2013) Protease-activated receptor 2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. The Journal of biological chemistry 288:5790-5802.

Popik P, Kozela E, Danysz W (2000) Clinically available NMDA receptor antagonists memantine and dextromethorphan reverse existing tolerance to the antinociceptive effects of morphine in mice. Naunyn-Schmiedeberg's archives of pharmacology 361:425-432.

Premkumar LS, Abooj M (2013) TRP channels and analgesia. Life sciences 92:415-424.

Premkumar LS, Ahern GP (2000) Induction of vanilloid receptor channel activity by protein kinase C. Nature 408:985-990.

Qin X, Wan Y, Wang X (2005) CCL2 and CXCL1 trigger calcitonin gene-related peptide release by exciting primary nociceptive neurons. Journal of neuroscience research 82:51-62.

Rao RP, Sejnowski TJ (2001) Spike-timing-dependent Hebbian plasticity as temporal difference learning. Neural computation 13:2221-2237.

Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M (2002) PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:4740-4745.

Reed DE, Barajas-Lopez C, Cottrell G, Velazquez-Rocha S, Dery O, Grady EF, Bunnett NW, Vanner SJ (2003) Mast cell tryptase and proteinase-activated receptor 2 induce hyperexcitability of guinea-pig submucosal neurons. The Journal of physiology 547:531-542.

Reeves BN, Dakhil SR, Sloan JA, Wolf SL, Burger KN, Kamal A, Le-Lindqwister NA, Soori GS, Jaslowski AJ, Kelaghan J, Novotny PJ, Lachance DH, Loprinzi CL (2012) Further data supporting that paclitaxel-associated acute pain syndrome is associated with development of peripheral neuropathy: North Central Cancer Treatment Group trial N08C1. Cancer 118:5171-5178.

Resman N, Gradisar H, Vasl J, Keber MM, Pristovsek P, Jerala R (2008) Taxanes inhibit human TLR4 signaling by binding to MD-2. FEBS letters 582:3929-3934.

Rexed B (1952) The cytoarchitectonic organization of the spinal cord in the cat. The Journal of comparative neurology 96:414-495.

Rexed B, Strom G (1952) Afferent nervous connexions of the lateral cervical nucleus. Acta physiologica Scandinavica 25:219-229.

Reynolds DV (1969) Surgery in the rat during electrical analgesia induced by focal brain stimulation. Science 164:444-445.

Rivera-Arconada I, Lopez-Garcia JA (2010) Changes in membrane excitability and potassium currents in sensitized dorsal horn neurons of mice pups. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:5376-5383.

Rothmeier AS, Ruf W (2012) Protease-activated receptor 2 signaling in inflammation. Seminars in immunopathology 34:133-149.

Saifeddine M, al-Ani B, Cheng CH, Wang L, Hollenberg MD (1996) Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue. British journal of pharmacology 118:521-530.

Saito S, Tominaga M (2015) Functional diversity and evolutionary dynamics of thermoTRP channels. Cell Calcium 57:214-221.

Sandkuhler J (2009) Models and mechanisms of hyperalgesia and allodynia. Physiological reviews 89:707-758.

Sawada K, Nishibori M, Nakaya N, Wang Z, Saeki K (2000) Purification and characterization of a trypsin-like serine proteinase from rat brain slices that degrades laminin and type IV collagen and stimulates protease-activated receptor-2. Journal of neurochemistry 74:1731-1738.

Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH (2009) Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462:651-655.

Seatter MJ, Drummond R, Kanke T, Macfarlane SR, Hollenberg MD, Plevin R (2004) The role of the C-terminal tail in protease-activated receptor-2-mediated Ca2+ signalling, proline-rich tyrosine kinase-2 activation, and mitogen-activated protein kinase activity. Cellular signalling 16:21-29.

Seeliger S, Derian CK, Vergnolle N, Bunnett NW, Nawroth R, Schmelz M, Von Der Weid PY, Buddenkotte J, Sunderkotter C, Metze D, Andrade-Gordon P, Harms E, Vestweber D, Luger TA, Steinhoff M (2003) Proinflammatory role of proteinase-activated receptor-2 in humans and mice during cutaneous inflammation in vivo. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17:1871-1885.

Shikamoto Y, Morita T (1999) Expression of factor X in both the rat brain and cells of the central nervous system. FEBS letters 463:387-389.

Shu X, Mendell LM (1999) Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neuroscience letters 274:159-162.

Scholz J, Woolf CJ (2002) Can we conquer pain? Nature neuroscience 5 Suppl:1062-1067.

Simone DA, Baumann TK, LaMotte RH (1989) Dose-dependent pain and mechanical hyperalgesia in humans after intradermal injection of capsaicin. Pain 38:99-107.

Simone DA, Nolano M, Johnson T, Wendelschafer-Crabb G, Kennedy WR (1998) Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers: correlation with sensory function. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:8947-8959.

Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L, Egerton J, Charles KJ, Smart D, Randall AD, Anand P, Davis JB (2002) TRPV3 is a temperature-sensitive vanilloid receptor-like protein. Nature 418:186-190.

Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629-632.

Spicarova D, Adamek P, Kalynovska N, Mrozkova P, Palecek J (2014a) TRPV1 receptor inhibition decreases CCL2-induced hyperalgesia. Neuropharmacology 81:75-84.

Spicarova D, Nerandzic V, Palecek J (2011) Modulation of spinal cord synaptic activity by tumor necrosis factor alpha in a model of peripheral neuropathy. J Neuroinflammation 8:177.

Spicarova D, Nerandzic V, Palecek J (2014b) Update on the role of spinal cord TRPV1 receptors in pain modulation. Physiol Res 63 Suppl 1:S225-236.

Spicarova D, Palecek J (2008) The role of spinal cord vanilloid (TRPV1) receptors in pain modulation. Physiol Res 57 Suppl 3:S69-77.

Spicarova D, Palecek J (2009) The role of the TRPV1 endogenous agonist N-Oleoyldopamine in modulation of nociceptive signaling at the spinal cord level. Journal of neurophysiology 102:234-243.

Spicarova D, Palecek J (2010) Tumor necrosis factor alpha sensitizes spinal cord TRPV1 receptors to the endogenous agonist N-oleoyldopamine. J Neuroinflammation 7:49.

Spike RC, Todd AJ, Johnston HM (1993) Coexistence of NADPH diaphorase with GABA, glycine, and acetylcholine in rat spinal cord. The Journal of comparative neurology 335:320-333.

Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J (1987) Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proceedings of the National Academy of Sciences of the United States of America 84:2975-2979.

Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, Luger TA, Schmelz M (2003) Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:6176-6180.

Steinhoff M, Vergnolle N, Young SH, Tognetto M, Amadesi S, Ennes HS, Trevisani M, Hollenberg MD, Wallace JL, Caughey GH, Mitchell SE, Williams LM, Geppetti P, Mayer EA, Bunnett NW (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nature medicine 6:151-158.

Stokes JA, Corr M, Yaksh TL (2013) Spinal toll-like receptor signaling and nociceptive processing: regulatory balance between TIRAP and TRIF cascades mediated by TNF and IFNbeta. Pain 154:733-742.

Striggow F, Riek-Burchardt M, Kiesel A, Schmidt W, Henrich-Noack P, Breder J, Krug M, Reymann KG, Reiser G (2001) Four different types of protease-activated receptors are widely expressed in the brain and are up-regulated in hippocampus by severe ischemia. The European journal of neuroscience 14:595-608.

Suen JY, Cotterell A, Lohman RJ, Lim J, Han A, Yau MK, Liu L, Cooper MA, Vesey DA, Fairlie DP (2014) Pathway-selective antagonism of proteinase activated receptor 2. British journal of pharmacology 171:4112-4124.

Suen JY, Gardiner B, Grimmond S, Fairlie DP (2010) Profiling gene expression induced by protease-activated receptor 2 (PAR2) activation in human kidney cells. PLoS One 5:e13809.

Sugiura T, Tominaga M, Katsuya H, Mizumura K (2002) Bradykinin lowers the threshold temperature for heat activation of vanilloid receptor 1. J Neurophysiol 88:544-548.

Sun JH, Yang B, Donnelly DF, Ma C, LaMotte RH (2006) MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. J Neurophysiol 96:2189-2199.

Szajnik M, Szczepanski MJ, Czystowska M, Elishaev E, Mandapathil M, Nowak-Markwitz E, Spaczynski M, Whiteside TL (2009) TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. Oncogene 28:4353-4363.

Szallasi A, Blumberg PM (1991) Characterization of vanilloid receptors in the dorsal horn of pig spinal cord. Brain research 547:335-338.

Szallasi A, Blumberg PM, Nilsson S, Hokfelt T, Lundberg JM (1994) Visualization by [3H]resiniferatoxin autoradiography of capsaicin-sensitive neurons in the rat, pig and man. European journal of pharmacology 264:217-221.

Szallasi A, Nilsson S, Farkas-Szallasi T, Blumberg PM, Hokfelt T, Lundberg JM (1995) Vanilloid (capsaicin) receptors in the rat: distribution in the brain, regional differences in the spinal cord, axonal transport to the periphery, and depletion by systemic vanilloid treatment. Brain research 703:175-183.

Takazawa T, MacDermott AB (2010) Glycinergic and GABAergic tonic inhibition fine tune inhibitory control in regionally distinct subpopulations of dorsal horn neurons. The Journal of physiology 588:2571-2587.

Talavera K, Yasumatsu K, Voets T, Droogmans G, Shigemura N, Ninomiya Y, Margolskee RF, Nilius B (2005) Heat activation of TRPM5 underlies thermal sensitivity of sweet taste. Nature 438:1022-1025.

Tanaka T, Minami M, Nakagawa T, Satoh M (2004) Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. Neuroscience research 48:463-469.

Thacker MA, Clark AK, Bishop T, Grist J, Yip PK, Moon LD, Thompson SW, Marchand F, McMahon SB (2009) CCL2 is a key mediator of microglia activation in neuropathic pain states. Eur J Pain 13:263-272.

Thayer SA, Perney TM, Miller RJ (1988) Regulation of calcium homeostasis in sensory neurons by bradykinin. The Journal of neuroscience : the official journal of the Society for Neuroscience 8:4089-4097.

Thompson SW, Woolf CJ, Sivilotti LG (1993) Small-caliber afferent inputs produce a heterosynaptic facilitation of the synaptic responses evoked by primary afferent A-fibers in the neonatal rat spinal cord in vitro. J Neurophysiol 69:2116-2128.

Tillu DV, Hassler SN, Burgos-Vega CC, Quinn TL, Sorge RE, Dussor G, Boitano S, Vagner J, Price TJ (2015) Protease-activated receptor 2 activation is sufficient to induce the transition to a chronic pain state. Pain 156:859-867.

Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. Nature reviews Neuroscience 11:823-836.

Togashi K, Hara Y, Tominaga T, Higashi T, Konishi Y, Mori Y, Tominaga M (2006) TRPM2 activation by cyclic ADP-ribose at body temperature is involved in insulin secretion. The EMBO journal 25:1804-1815.

Tohda C, Sasaki M, Konemura T, Sasamura T, Itoh M, Kuraishi Y (2001) Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. Journal of neurochemistry 76:1628-1635.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531-543.

Tominaga M, Tominaga T (2005) Structure and function of TRPV1. Pflugers Archiv : European journal of physiology 451:143-150.

Tominaga M, Wada M, Masu M (2001) Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proceedings of the National Academy of Sciences of the United States of America 98:6951-6956.

Touska F, Marsakova L, Teisinger J, Vlachova V (2011) A "cute" desensitization of TRPV1. Curr Pharm Biotechnol 12:122-129.

Tracey I, Mantyh PW (2007) The cerebral signature for pain perception and its modulation. Neuron 55:377-391.

Treede RD, Meyer RA, Raja SN, Campbell JN (1992) Peripheral and central mechanisms of cutaneous hyperalgesia. Progress in neurobiology 38:397-421.

Ueda M, Kuraishi Y, Satoh M (1993) Detection of capsaicin-evoked release of glutamate from spinal dorsal horn slices of rat with on-line monitoring system. Neuroscience letters 155:179-182.

Uchytilova E, Spicarova D, Palecek J (2014) TRPV1 antagonist attenuates postoperative hypersensitivity by central and peripheral mechanisms. Mol Pain 10:67.

Valtschanoff JG, Rustioni A, Guo A, Hwang SJ (2001) Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. The Journal of comparative neurology 436:225-235.

Van Steenwinckel J, Reaux-Le Goazigo A, Pommier B, Mauborgne A, Dansereau MA, Kitabgi P, Sarret P, Pohl M, Melik Parsadaniantz S (2011) CCL2 released from neuronal synaptic vesicles in the spinal cord is a major mediator of local inflammation and pain after peripheral nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:5865-5875.

Vellani V, Mapplebeck S, Moriondo A, Davis JB, McNaughton PA (2001) Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. The Journal of physiology 534:813-825.

Vellani V, Zachrisson O, McNaughton PA (2004) Functional bradykinin B1 receptors are expressed in nociceptive neurones and are upregulated by the neurotrophin GDNF. The Journal of physiology 560:391-401.

Vergnolle N (2000) Review article: proteinase-activated receptors - novel signals for gastrointestinal pathophysiology. Alimentary pharmacology & therapeutics 14:257-266.

Vergnolle N (2009) Protease-activated receptors as drug targets in inflammation and pain. Pharmacology & therapeutics 123:292-309.

Vergnolle N, Bunnett NW, Sharkey KA, Brussee V, Compton SJ, Grady EF, Cirino G, Gerard N, Basbaum AI, Andrade-Gordon P, Hollenberg MD, Wallace JL (2001a) Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway. Nature medicine 7:821-826.

Vergnolle N, Hollenberg MD, Sharkey KA, Wallace JL (1999) Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. British journal of pharmacology 127:1083-1090.

Vergnolle N, Wallace JL, Bunnett NW, Hollenberg MD (2001b) Protease-activated receptors in inflammation, neuronal signaling and pain. Trends in pharmacological sciences 22:146-152.

Vlachova V, Teisinger J, Susankova K, Lyfenko A, Ettrich R, Vyklicky L (2003) Functional role of C-terminal cytoplasmic tail of rat vanilloid receptor 1. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:1340-1350.

Volk-Draper L, Hall K, Griggs C, Rajput S, Kohio P, DeNardo D, Ran S (2014) Paclitaxel therapy promotes breast cancer metastasis in a TLR4-dependent manner. Cancer research 74:5421-5434.

Vriens J, Owsianik G, Hofmann T, Philipp SE, Stab J, Chen X, Benoit M, Xue F, Janssens A, Kerselaers S, Oberwinkler J, Vennekens R, Gudermann T, Nilius B, Voets T (2011) TRPM3 is a nociceptor channel involved in the detection of noxious heat. Neuron 70:482-494.

Wall PD, McMahon SB, Koltzenburg M (2006) Wall and Melzack's textbook of pain, 5th ed. Edition. Philadelphia: Elsevier/Churchill Livingstone.

Wang DO, Kim SM, Zhao Y, Hwang H, Miura SK, Sossin WS, Martin KC (2009) Synapse- and stimulus-specific local translation during long-term neuronal plasticity. Science 324:1536-1540.

Wang H, Ubl JJ, Reiser G (2002a) Four subtypes of protease-activated receptors, co-expressed in rat astrocytes, evoke different physiological signaling. Glia 37:53-63.

Wang J, Kobayashi M, Han M, Choi S, Takano M, Hashino S, Tanaka J, Kondoh T, Kawamura K, Hosokawa M (2002b) MyD88 is involved in the signalling pathway for Taxol-induced apoptosis and TNF-alpha expression in human myelomonocytic cells. British journal of haematology 118:638-645.

Wang S, Dai Y, Fukuoka T, Yamanaka H, Kobayashi K, Obata K, Cui X, Tominaga M, Noguchi K (2008) Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain. Brain : a journal of neurology 131:1241-1251.

Wang Y, Kedei N, Wang M, Wang QJ, Huppler AR, Toth A, Tran R, Blumberg PM (2004) Interaction between protein kinase Cmu and the vanilloid receptor type 1. The Journal of biological chemistry 279:53674-53682.

Wei H, Wei Y, Tian F, Niu T, Yi G (2016) Blocking proteinase-activated receptor 2 alleviated neuropathic pain evoked by spinal cord injury. Physiol Res 65:145-153.

Weiner RSPD (2002) Pain management : a practical guide for clinicians, 6th ed. /editor, Richard S. Weiner. Edition. Boca Raton ; London: CRC Press.

White CM, Martin BK, Lee LF, Haskill JS, Ting JP (1998) Effects of paclitaxel on cytokine synthesis by unprimed human monocytes, T lymphocytes, and breast cancer cells. Cancer immunology, immunotherapy : CII 46:104-112.

White FA, Sun J, Waters SM, Ma C, Ren D, Ripsch M, Steflik J, Cortright DN, Lamotte RH, Miller RJ (2005) Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. Proceedings of the National Academy of Sciences of the United States of America 102:14092-14097.

Wiegand U, Corbach S, Minn A, Kang J, Muller-Hill B (1993) Cloning of the cDNA encoding human brain trypsinogen and characterization of its product. Gene 136:167-175.

Willis WD, Coggeshall RE (2004) Sensory mechanisms of the spinal cord, 3rd ed. Edition. New York ; London: Kluwer Academic/Plenum.

Woolf CJ (1991) Generation of acute pain: central mechanisms. British medical bulletin 47:523-533.

Woolf CJ (2010) What is this thing called pain? The Journal of clinical investigation 120:3742-3744.

Woolf CJ (2011) Central sensitization: implications for the diagnosis and treatment of pain. Pain 152:S2-15.

Woolf CJ, King AE (1989) Subthreshold components of the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat lumbar spinal cord. J Neurophysiol 62:907-916.

Woolf CJ, King AE (1990) Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 10:2717-2726.

Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. Neuron 55:353-364.

Woolf CJ, Mannion RJ (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353:1959-1964.

Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. Science 288:1765-1769.

Woolf CJ, Shortland P, Sivilotti LG (1994) Sensitization of high mechanothreshold superficial dorsal horn and flexor motor neurones following chemosensitive primary afferent activation. Pain 58:141-155.

Wu Y, Liu Y, Hou P, Yan Z, Kong W, Liu B, Li X, Yao J, Zhang Y, Qin F, Ding J (2013) TRPV1 channels are functionally coupled with BK(mSlo1) channels in rat dorsal root ganglion (DRG) neurons. PLoS One 8:e78203.

Wu ZZ, Chen SR, Pan HL (2006) Signaling mechanisms of down-regulation of voltage-activated Ca2+ channels by transient receptor potential vanilloid type 1 stimulation with olvanil in primary sensory neurons. Neuroscience 141:407-419.

Yamada KA, Tang CM (1993) Benzothiadiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. The Journal of neuroscience : the official journal of the Society for Neuroscience 13:3904-3915.

Yamasaki R, Liu L, Lin J and Ransohoff R.M (2011) Role of CCR2 in immunobiology and neurobiology Clinical and Experimental Neuroimmunology 3 (2012) 16–29

Yao H, Peng F, Dhillon N, Callen S, Bokhari S, Stehno-Bittel L, Ahmad SO, Wang JQ, Buch S (2009) Involvement of TRPC channels in CCL2-mediated neuroprotection against tat toxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:1657-1669.

Yau MK, Liu L, Fairlie DP (2013) Toward drugs for protease-activated receptor 2 (PAR2). Journal of medicinal chemistry 56:7477-7497.

Yuan H, Zhu X, Zhou S, Chen Q, Ma X, He X, Tian M, Shi X (2010) Role of mast cell activation in inducing microglial cells to release neurotrophin. Journal of neuroscience research 88:1348-1354.

Zhang H, Dougherty PM (2014) Enhanced excitability of primary sensory neurons and altered gene expression of neuronal ion channels in dorsal root ganglion in paclitaxel-induced peripheral neuropathy. Anesthesiology 120:1463-1475.

Zhang J, De Koninck Y (2006) Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. Journal of neurochemistry 97:772-783.

Zhang J, Shi XQ, Echeverry S, Mogil JS, De Koninck Y, Rivest S (2007) Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. The

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Journal of neuroscience : the official journal of the Society for Neuroscience 27:12396-12406.

Zhang ZJ, Dong YL, Lu Y, Cao S, Zhao ZQ, Gao YJ (2012) Chemokine CCL2 and its receptor CCR2 in the medullary dorsal horn are involved in trigeminal neuropathic pain. J Neuroinflammation 9:136.

Zhao P, Metcalf M, Bunnett NW (2014) Biased signaling of protease-activated receptors. Frontiers in endocrinology 5:67.

Zhu W, Oxford GS (2007) Phosphoinositide-3-kinase and mitogen activated protein kinase signaling pathways mediate acute NGF sensitization of TRPV1. Molecular and cellular neurosciences 34:689-700.

Zimmer SM, Liu J, Clayton JL, Stephens DS, Snyder JP (2008) Paclitaxel binding to human and murine MD-2. The Journal of biological chemistry 283:27916-27926.

Zimmermann K, Lennerz JK, Hein A, Link AS, Kaczmarek JS, Delling M, Uysal S, Pfeifer JD, Riccio A, Clapham DE (2011) Transient receptor potential cation channel, subfamily C, member 5 (TRPC5) is a cold-transducer in the peripheral nervous system. Proceedings of the National Academy of Sciences of the United States of America 108:18114-18119.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D, Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400:452-457.

Zylka MJ, Rice FL, Anderson DJ (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45:17-25.

11.ATTACHMENT – original full text articles

Publication 1

TRPV1 receptor inhibition decreases CCL2-induced hyperalgesia.

Autors: Spicarova D, Adamek P, Kalynovska N, **Mrozkova P**, Palecek J.

The extent of participation: Preparation of the electrophysiological experiments, data analysis

Publication 2

The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4.

Autors: Li Y, Adamek P, Zhang H, Tatsui CE, Rhines LD, **Mrozkova P**, Li Q, Kosturakis AK, Cassidy RM, Harrison DS, Cata JP, Sapire K, Zhang H, Kennamer-Chapman RM, Jawad AB, Ghetti A, Yan J, Palecek J, Dougherty PM.

The extent of participation: Preparation part of the electrophysiological experiments, data analysis

Publication 3

The role of protease-activated receptor type 2 in nociceptive signaling and pain

Autors: **Mrozkova P**, Palecek J, Spicarova D.

The extent of participation: Preparation and writing of the manuscript (review)**.**

Publication 4

Hypersensitivity Induced by Activation of Spinal Cord PAR2 Receptors Is Partially Mediated by TRPV1 Receptors.

Autors: **Mrozkova P**, Spicarova D, Palecek J.

The extent of participation: Preparation of all behavioral and electrophysiological experiments, data analysis and writing of the manuscript.