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Bc. Dominika Šulcová

The role of spinal TRPV1 receptors in nociceptive signalling and the modulatory effect of chemokine CCL2 and μ-opioid receptor agonists

Úloha míšních TRPV1 receptorů v nociceptivním přenosu a modulační účinky chemokinu CCL2 a agonistů μ-opioidního receptoru

Diploma thesis

Supervisor: Jiří Paleček M.D., Ph.D.
Institute of Physiology AS CR
Department of Functional Morphology

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PROHLÁŠENÍ

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně na základě konzultací se školitelem a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 5. 2. 2017 Dominika Šulcová

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ABSTRAKT

První synapse nociceptivní dráhy v zadním rohu míšním představuje významné místo pro modulaci nociceptivní signalizace za patologických stavů. Jedním z modulačních mechanizmů jsou změny funkce receptoru TRPV1 (*transient receptor potential vanilloid 1*), který je exprimován na centrálních zakončeních nociceptivních neuronů a svou činností přímo reguluje výlev neuropřenašečů a neuromodulátorů. Předchozí studie ukázaly úlohu TRPV1 v účincích zánětlivého chemokinu CCL2 (*C-C motif ligand 2*) a po aktivaci μ-opioidního receptoru (MOP-R) na míšní úrovni. Protože receptory CCR2 (aktivovány CCL2), TRPV1 a MOP-R často kolokalizují v neuronech spinálních ganglií, cílem tohoto projektu bylo studovat jejich možné intreakce při modulaci nociceptivního přenosu v míše. Tato diplomová práce se zabývala zejména vlivem chemokinu CCL2 na senzitizaci spinálních TRPV1 u modelu periferní neuropatie a jeho vlivem na synaptický přenos po aktivaci μ-opioidních receptorů.

Ke studiu synaptického přenosu bylo využito snímání excitačních postsynaptických proudů (EPSC) pomocí metody *patch-clamp* z povrchových neuronů zadního rohu míšního na řezech lumbární míchy potkana. U těchto neuronů byla v závěru experimentu prokázána přítomnost TRPV1 pozitivních synapsí pomocí aplikace kapsaicinu. V první části experimentu byla měření prováděna na neuronech izolovaných ze zvířat s experimentálně navozenou periferní neuropatií. Zde došlo ke zvýšení frekvence mEPSC po podání TRPV1 agonisty OLDA (N-oleoyl dopamin) v nízké koncentraci (0.2 uM), která je u naivních neuronů neúčinná. Tím byla potvrzena senzitizace TRPV1 receptorů za podmínek periferní neuropatie. V neuronech z neuropatických zvířat inkubovaných s CCL2 byla pozorována vyšší bazální frekvence i amplitudy EPSC, a také silnější reakce na aplikaci OLDA, která však byla vysoce variabilní u jednotlivých měřených buněk. Druhá část experimentu byla prováděna na neuronech izolovaných z naivních zvířat, kde byl testován vliv agonisty MOP-R (D-Ala2,N-Me-Phe4,Gly-ol 5)-enkephalin (DAMGO). V kontrolní skupině byl pozorován dlouhotrvající inhibiční účinek aktivace MOP-R, který se projevil na frekvenci, a nikoliv na amplitudě mEPSC. Předchozí inkubace s CCL2 vedla ke snížení účinku DAMGO na frekvenci mEPSC, zatímco akutní aplikace CCL2 měla mnohem slabší efekt. Naše výsledky naznačují, že TRPV1, CCL2 a MOP-R mohou interagovat na aferentních zakončeních v první nociceptivní synapsi v zadním rohu míšním, pravděpodobně díky aktivaci různých nitrobuněčných mechanizmů, čímž mohou výrazně ovlivňovat modulaci nociceptivní signalizace za patologických stavů.

Klíčová slova: TRPV1, CCL2, μ-opioidní receptor, OLDA, DAMGO, zadní roh míšní, nocicepce

ABSTRACT

The first nociceptive synapse in the spinal cord dorsal horn represents an important site, where nociceptive synaptic transmission can be modulated under pathological conditions. One of the modulatory mechanism involves activation of the transient receptor potential vanilloid 1 (TRPV1) that is expressed on central terminals of primary nociceptive neurons, where it regulates release of neurotransmitters and neuromodulators. Previous studies suggested that changes in TRPV1 activity may be related to effects of chemokine CCL2 (C-C motif ligand 2) and may be also involved in synaptic transmission modulation after μ-opioid receptors (MOP-R) activation. Because CCL2 receptors CCR2 often co-localize with TRPV1 and MOP-R, the goal of this work was to study possible interactions of these receptors on the pre-synaptic endings of primary afferents in the spinal cord dorsal horn and their role in nociceptive signalling under pathological conditions. The presented thesis focused on the effect of CCL2 during peripheral neuropathy and its interference with μ-opioid receptor activation.

To study synaptic transmission at the spinal cord level, patch-clamp recordings of excitatory post-synaptic currents (EPSC) in superficial spinal cord dorsal horn neurons in acute lumbar spinal cord slices from rats was used. TRPV1-expressing afferents were confirmed by increased mEPSC frequency after capsaicin application in most of the neurons. In the first block of experiments, the recordings were made in neurons isolated from animals with peripheral neuropathy. We observed an increase in mEPSC frequency after application of TRPV1 agonist OLDA in a low, normally ineffective concentration (0.2 uM). This indicated that spinal TRPV1 receptors are sensitized during peripheral neuropathy. In neurons from neuropathic animals incubated with CCL2, we observed higher basal EPSC frequency and amplitude, as well as the response to OLDA that was, however, very diversified. The second block of experiments was conducted on neurons from naïve animals and the effect of MOP-R agonist (D-Ala2,N-Me-Phe4,Gly-ol 5)-enkephalin (DAMGO) application on synaptic transmission was tested. In the control group, we observed a long-lasting inhibitory effect of MOP-R activation that influenced the frequency but not the amplitude of mEPSC. In slices pre-incubated with CCL2, the inhibitory effect of DAMGO was partially diminished, suggesting a possible interaction between the receptors. Acute application of CCL2 before DAMGO had a much smaller effect. Our data indicate that TRPV1, CCL2 and MOP-R may interact at the first nociceptive synapse in the spinal cord dorsal horn, possibly through activation of distinct intracellular pathways, and may influence nociceptive transmission under pathological conditions.

Key words: TRPV1, CCL2, μ-opioid receptor, OLDA, DAMGO, spinal cord dorsal horn, nociception

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LIST OF ABBREVIATIONS

AA	amino acid
AC	adenylate cyclase
AEA	anandamide
AKAP79	A kinase anchoring protein 79
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BK	bradykinin
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin dependent kinase II
cAMP	cyclic adenosine monophosphate
CC/CXC/CX3C	chemokine families
CCD	chronic constriction of dorsal root ganglion
CCI	chronic constriction injury
CCL2	chemokine CC motif ligand 2
CCR2	receptor for chemokine CCL2
Cdk5	cyclin-dependent kinase 5
CGRP	calcitonin-gene related peptide
CNS	central nervous system
DAG	diacylglycerol
DAMGO	(D-Ala2,N-Me-Phe4,Gly-ol 5)-enkephalin
DH	dorsal horn
DMSO	dimethyl sulfoxide
DOP-R	δ-opioid receptor
DRG	dorsal root ganglion
DkTx	double knot toxin
ECS	extracellular solution
EGTA	ethylene glycol-bis(β-amino ethyl ether)-N,N,N',N'-tetra acetic acid
ERK	extracellular signal-regulated kinase
FRET	fluorescence resonance energy transfer
FRAP	Fluorescence recovery after photobleaching
GABA	gamma-aminobutyric acid
GDP	guanosine diphosphate
GPCR	G protein–coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-[gamma-thio] triphosphate
HEPES	4-(2-hydroxyethyl) iperazinethansulfonic acid
HPETE	hydroperoxyeicosatetraenoic acid
ICK	inhibitor cysteine knot domain
KOP-R	κ-opioid receptor
LTP	long-term potentiation
MCP-1	monocyte chemoattractant protein-1
MOP-R	μ-opioid receptor
MrgprD	Mas-related G protein-coupled receptor member D
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NK_1	neurokinin
NK_1R	neurokinin receptor 1
NMDA	N-methyl-D-aspartate
NADA	N-arachidonoyl-dopamine
OIH	opioid-induced hyperalgesia
OLDA	N-oleoyl-dopamine
OLEA	N-oleoyl-ethanolamine

$P2X_3$	purinergic receptor
$P2Y_1$	purinergic receptor
PAF	primary afferent fiber
PG	prostaglandin
PGE_2/I_2	prostaglandin E_2/I_2
pH	potential of hydrogen
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PNL/PSNL	partial sciatic nerve ligation
POMC	proopiomelanocortin
RTX	resiniferatoxin
RT-PCR	transcription-polymerase chain reaction
s/eIPSC	spontaneous/evoked inhibitory postsynaptic current
s/e/mEPSC	spontaneous/evoked/miniature excitatory postsynaptic current
SEM	standard error of mean
SNI	spared nerve injury model
SNL	spinal nerve ligation
SP	substance P
TNFα	tumor necrosis factor α
TrkA	nerve growth factor receptor
TRP	transient receptor potential
TRPA	TRP ankyrin family
TRPM	TRP melastatin family
TRPV1	TRP vanilloid 1
TTX	tetrodotoxin
VaTx	vanillotoxin
WDR	wide dynamic range neuron

THEORETICAL PART

1. INTRODUCTION

The perception of pain is unpleasant both physically and emotionally, but that doesn't mean it is necessarily bad. On the contrary, the ability to feel pain has developed as a priceless tool that helps humans and some animals to detect, and even predict an injury or illness. The pain as a physiological warning occurs as a result of nociceptive signalling that is mediated by a network of specialized sensory neurons. Under specific conditions, any component of this network – from peripheral receptors to cortical processing centres – might malfunction and initiate changes in nociceptive signalling that manifest as pathological neuropathic pain.

This work concentrates on pathologies affecting dorsal root ganglion (DRG) neurons and more specifically on the changes that take place at their central terminals, primary afferent fibers. The first synapse of the nociceptive pathway is located in the dorsal horn of the spinal cord and it represents a major site of nociceptive modulation, where the strength and character of the afferent signal can be modified or completely attenuated according to current conditions. Many recent studies suggest that plastic changes occurring at the first synapse represent the basis for the development and maintenance of chronic neuropathic pain (Luo et al. 2014; West et al. 2015). At the same time, the spinal cord is relatively accessible with a well-known structure. Therefore it seems that the first synapse might be an ideal place for analgesic intervention.

Primary afferent fibers that specialize in nociceptive signalling comprise of several populations that can be distinguished by different sets of expressed proteins and receptors. One of the molecules playing a significant role in nociception is transient receptor potential vanilloid 1 (TRPV1), a nonselective cation channel predominantly found in a population of small diameter fibers that are responsible for transduction and transmission of noxious heat (Cui et al. 2016). In peripheral nociceptive endings, TRPV1 works as a molecular integrator of nociceptive stimuli and ensures the ability to register high temperatures (Kissin and Szallasi 2011). TRPV1 is also expressed on central terminals of primary afferent fibers and its function there is much less clear (Spicarova and Palecek 2008). However, it has been reported that increased activation of spinal TRPV1 results in enhanced synaptic transmission at the first nociceptive synapse and changes in TRPV1 activity have been associated with several pathological pain states, which suggests that it might play an important role in development and maintenance of nociceptive signalling associated with chronic pain (Palazzo et al. 2012; Spicarova et al. 2014).

Various endogenous substances can contribute to increased TRPV1 activation including some of the inflammatory mediators produced by neurons and neighbouring glial and immune cells under pathological conditions. Amongst these, inflammatory chemokines occupy a prominent

position – they are no longer considered as mere signalling molecules of the immune system, they have been recognized as mediators of the bidirectional communication between neuronal and non-neuronal cells that may contribute to pathological pain conditions (Rostène et al., 2007). Chemokine CCL2 in particular is in the centre of our attention, since its pro-nociceptive action associated with modulation of presynaptic TRPV1 receptor (Gao et al. 2009; Spicarova et al. 2014).

On the other side stand substances that moderate the spinal TRPV1 activity and growing evidence suggests that this might be the nature of opioid receptors activation, which in most cases contributes to pain attenuation (Bao et al. 2015; Endres-Becker et al. 2007; Vetter et al. 2008). In fact, opioid analgesics are still considered as the most effective drugs for treatment of severe chronic pain and their use increases in spite of their serious side effects (Kissin 2010). Surprisingly, in case of neuropathic pain, opioids often come short and very high doses of the drug are required to achieve sufficient relief, which indicates that the specific conditions during neuropathic pain state employ different mechanisms not easily diminished with opioid action (Arner and Meyerson 1988; Wright 1994). The first nociceptive synapse in the spinal cord represents the principal site of opioid action (Chen and Pan 2006a; Yaksh and Noueihed 1985) and it is also one of the places where the opioid system has the opportunity to interact with the chemokine system. Together these two major players contribute to the environment that also modulates presynaptic TRPV1 activity. However, to this day very little is known about the character of opioid-chemokine interaction and its effect on TRPV1 at the spinal cord level (Parsadaniantz et al. 2015).

This work explores the effect of chemokine CCL2 on nociceptive transmission between TRPV1-positive primary afferents and secondary neurons in the superficial dorsal horn of the spinal cord (1) in animal model of peripheral neuropathy and (2) with simultaneous application of μ-opioid receptor agonist (D-Ala2,N-Me-Phe4,Gly-ol 5)-enkephalin (DAMGO). Although the results presented in the experimental part of this thesis are preliminary and more extensive research is needed, they represent the first step in a promising direction. Detailed knowledge of the processes that regulate nociceptive signalling in the spinal cord under pathological conditions might in the future help identify optimal therapeutic targets, facilitate the development of new efficient drugs and thus improve the management of chronic neuropathic pain.

2. NOCICEPTIVE SIGNALLING

2.1. Nociception and pain

The expression ‘pain’ describes an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage, as defined by the Internationa Association for the Study of Pain (IASP 2015). This definition indicates that pain perception is useful to an individual for many reasons: it warns the organism of an existing injury, prevents self-harming, and the increased sensitivity to normally innocuous stimuli protects the healing tissue from being further disturbed. The term ‘nociception’ is not synonymous with pain, as it describes the very process of transformation of a sensory input into a neurological signal, which is consequently transmitted along neural pathways to the brain. Nociceptive information can, but does not necessarily have to be translated into painful experience and similarly painful feeling can occur without any nociceptive input (Lee et al. 2009).

Nociceptive processes are triggered by a noxious stimulus that is detected by peripheral endings of specialized primary sensory neurons, and if the stimulus intensity surpasses a set threshold, the sensory neurons generate an afferent signal. Such threshold is not fixed, it can be shifted through diverse molecular mechanisms according to the current level of neuronal activity, thus modifying the response to a stimulus. At every stage of the sensory pathway there is a threshold to pass, and so every synapse represents a site of signal modulation that contributes to neural plasticity. Synaptic changes might result either in adaptation and attenuation of pain or, conversely, in hyper excitation possibly leading to the development of a pathological pain syndrome. Patients suffering from chronic pain of the same etiology usually show similar plastic changes in nociceptive transmission but they can be greatly diversified regarding to the level of pain they experience, because the cortical processing of a nociceptive signal is very subjective and so every individual perceives pain differently (Scholz and Woolf 2002).

Pain, as negative experience, can be categorized by various aspects. When categorized by pathophysiological mechanism, we can distinguish nociceptive pain, as it is described above, inflammatory pain and neuropathic pain. Inflammatory pain occurs as a response of primary sensory neurons to binding of various inflammatory mediators (such as histamine, serotonin, bradykinin, prostaglandins, interleukins, ATP, H^+ and many other) which are produced by cells of affected tissue and immune cells during inflammation. By contrast, neuropathic pain is not primarily related to pathology of innervated tissue but is caused by damage of nerve tissue itself, either peripheral or central. This specific type of pain will be further described in chapter 3.2 (Scholz and Woolf 2002).

Other common system of classification describes various types of pain according to the length of pain duration. Acute pain is sudden, sharp and immediately following an injury. It is physiological nociceptive pain which usually lasts less than 30 days and disappears after the injury heals. Conversely, chronic pain is described as pain lasting 3 to 6 months that is usually prolonged even after the time of the tissue regeneration or might occur without any visible cause, as it is usually caused by a complex mix of pathologies along the neural pathway. Such pain can be continuous or recurrent and in any case it can affect all aspects of daily life (Woessner 2006).

Pain is also characterized by the site of its origin. Somatic pain is generated in peripheral fibers innervating skin and muscles, it's usually intensive and well localized. Conversely, visceral pain comes from inner organs and is more diffuse. Furthermore, pain can be described by anatomic localization in specific segments and body parts.

2.2. Nociceptors and primary afferent fibers

It was established in 1960s that pain reception is mediated by its own system of specialized sensory cells. This class of primary sensory neurons was named nociceptors because of their ability to detect harmful and potentially damaging stimuli (mechanical, chemical and thermal) and to transform the information of their presence into nociceptive signals (Bessou et al. 1969). Individual nociceptors can detect one or multiple types of stimuli and employ various transduction mechanisms. They are able to integrate large variety of signals and subsequently react to dynamic changes in the environment (Julius and Basbaum 2001).

Like all primary sensory neurons, nociceptors are so-called pseudo unipolar cells, whose cell bodies reside in the dorsal root ganglion (DRG) or, in case of head innervation, in the trigeminal ganglion (TG). Their solitary axons forms a bifurcation, sending one terminal towards the peripheral target tissue and the other towards the central synapse in the dorsal horn of the spinal cord (DH) or in the pons, in case of TG neurons. According to Woolf and Ma, nociceptors constitute of four major functional departments: the peripheral ending that intercepts external stimuli and initiates action potentials, the cell body that ensures the integrity of the whole neuron, the axon that conducts the signal and the central ending that represents the presynaptic part of the first nociceptive synapse (Woolf and Ma 2007).

In DRG several separate populations of neurons can be found, with different cell body size, which corresponds with the diameter of the relevant fiber, while the conduction velocity of action

potentials in these fibers is also dependent on this diameter and on the level of axon myelination (Julius and Basbaum 2001). Receptors of innocuous stimuli have ordinarily large cell bodies and give rise to fast, thick (Ø >10 μm) myelinated Aβ fibers. Peripheral nociceptive fibers are thinner and belong to cell bodies of medium or small size that can be further divided into two distinctive groups – cells with non-myelinated axons (C fibers) and with thinly myelinated axons (Aδ fibers). The proportion of each receptor population to all primary sensory fibers in skin is approximately Aβ 20 %, A*δ* 10 % a C 70 %, which indicates high representation of nociceptors (Millan 1999).

Lightly myelinated Aδ fibers of medium diameter (2–6 μm) can conduct the signal as fast as 12–30 $m.sec^{-1}$ (in comparison, the fastest Aβ fibers conduct ~30–100 $m.sec^{-1}$) (Millan 1999). Apart from nociceptors, Aδ sensory fibers also include a population of low threshold D-hair receptors, a type of mechanoreceptors with the highest known sensitivity (Lewin and Moshourab 2004). High threshold Aδ nociceptors are called A-mechano-nociceptors, or free nerve endings, and they respond to mechanical, chemical and thermal noxious stimuli. They can be further divided into two groups, distinguishable by different reaction threshold or sensitivity to tissue injury. Type I Aδ fibers respond to high temperatures around 53 ° C but if the stimulus persists, they undergo substantial sensitization. Their response threshold is also lowered during presence of tissue damage. Comparatively, type II Aδ fibers have heat threshold relatively low (~ 43 ° C) and their mechanical threshold is much higher (Basbaum et al. 2009; Julius and Basbaum 2001).

C fibers are the thinnest (Ø = 0,4–1,2 μm) and they aren't myelinated at all, therefore the conduction of action potentials in these fibers is the slowest (~0,5–2 $m.sec^{-1}$) (Millan 1999). Most of these nociceptors are also polymodal – they are both heat and mechanically sensitive, therefore they are called C fiber mechano-heat nociceptive afferents (CMHs). Besides the former, C fibers nociceptors also include a population of so-called silent receptors, neurons responsive to heat but normally mechanically insensitive, which gain mechanical sensitivity only in presence of an injury. Silent receptors are also more sensitive to chemical stimuli (like capsaicin or histamine) compared to the CMHs, which supports the idea of them being set on by specific chemical milieu caused by inflammation (Basbaum et al. 2009).

Moreover, C fiber nociceptors can be divided into separate populations according to the type of chemical substance they release from their central terminals (Dubin and Patapoutian 2010). Some of these nociceptors are peptidergic, which means that they express (beside others) neuropeptide calcitonin gene-related peptide (CGRP) and substance P (SP). It's also typical for them to express a receptor TrkA that has high affinity to the nerve growth factor (NGF). In the embryonic stage, all new-born nociceptors are TrkA-positive but during the perinatal and postnatal stage, about a half of them switch off TrkA and start expressing Ret, which is a component of the

receptor for glia-derived neurotrophic factor (GDNF). These Ret positive cells become non-peptidergic nociceptors, characteristic by their ability to bind isolectin B4 (IB4) and the expression of $P2X_3$ receptor (a ion channel responding to ATP) (Woolf and Ma 2007). Interestingly, these two populations of C fiber nociceptors have also distinctive targets as the peripheral terminals of non-peptidergic nociceptors can be mostly found in epidermis while the peptidergic nociceptors innervate deeper regions of the skin as well as many other tissues (Todd 2010).

Because of different speed of signal conduction in each population of nociceptive fibers, two phases of pain sensation can be distinguished. The first, sharp pain is mediated by Aδ fibers and the second, delayed and more diffuse pain is mediated by C fibers. The first phase is missing in visceral pain that is typically hard to localize, deep and dull (Julius and Basbaum 2001).

All previously stated facts were taking in consideration the nociceptors innervating skin areas, whereas in other tissues the features of nociceptors can be much different. For example the nociception in dental pulp is mostly mediated by Aβ fibers that are known to respond even to light stimuli that are perceived as innocuous in skin (Chung et al. 2013). Visceral nociception is also specific as it often doesn't require any tissue damage (for example excessive distension might be sufficient) and it's characterized by high number of silent nociceptors, leading to substantial sensitization in the presence of inflammation (Keszthelyi et al. 2012). It is therefore quite difficult to describe a nociceptor only in terms of its response threshold or speed of signal conduction.

2.3. Nociceptive transmission at the spinal cord level

The dorsal horn (DH) of the spinal cord is the first site of integration of somatosensory information where the central terminals of DRG neurons form synapses with secondary spinal neurons. We can find there four basic neuronal units - primary afferent inputs, interneurons, projection neurons and descending modulating endings from the brain (Todd 2010). These neuronal units are engaged in complex dynamic communication, which is furthermore influenced by temporary chemical profile of extracellular environment.

The topography of the afferent ending in the DH is specific for every DRG neuron, while the rostro-caudal and medio-lateral position is determined by the location of the target tissue and the dorso-ventral position (the depth) reflects the functional identity of the neuron. It is a result of different axonal guidance during the neurogenesis for each population of DRG neurons (Woolf and Ma 2007). The gray matter of DH can be vertically split into 10 layers – so called laminae I – X

with characteristic neuronal structure and density (Rexed 1952). Low threshold sensory neurons with Aβ fibers are known to send their central terminals mostly to laminae IIi – V, whereas high threshold nociceptors form synapses in laminae I and II. Aδ fibers project into lamina I (except of the fibers innervating hair follicles that end between lamina II and III), the majority of peptidergic C fibers project into lamina I and the outer part of lamina IIo (a small part of them go also to deeper laminae) and non-peptidergic C fibers end predominantly in lamina IIi (Millan 1999; Todd 2010).

Nociceptors are excitatory neurons and they produce glutamate as their primary neurotransmitter, along with various neuropeptides (like SP and CGRP) and other substances that facilitate and modulate the transmission (like ATP, NO, growth factors, cytokines…). This chemical diversity leads to fast and slow postsynaptic evocation of action potentials that undergo temporal and spatial summation (Woolf and Ma 2007).

Facing the nociceptors, there are secondary neurons of DH and these can be in general categorized according to their response to nociceptive input. The first group comprises of nociceptive-specific (NS) neurons that are only activated by stimuli of high intensity, which are detected by Aδ and C fibers. These neurons are located in laminae I and II and are silent most of the time. The second group of DH neurons corresponds to so-called non-nociceptive (NON-N) neurons, which normally don't participate in nociceptive transmission and are located mainly in laminae II, III and IV. Lastly, there is a group of DH neurons called wide-dynamic range (WDR) neurons that form synapses with a broad range of primary afferents. The WDR neurons integrate inputs from receptors of different modalities as well as information of stimuli of different intensity, from very light and innocuous to noxious (Millan 1999). The WDR neurons also converge signals from both somatic and visceral receptors, which results in highly generalized information and can contribute to development of referred pain (Basbaum et al. 2009).

The synapses between primary nociceptive afferents and secondary neurons in the DH are not as simple as a one way stream of data, rather the opposite. They are in a close relationship with neighbouring glial cells that produce many neuromodulators, such as inflammatory cytokines and chemokines (like CX3CL1 or CCL2) (Gao and Ji 2010). Moreover, nociceptive transmission can be regulated in the DH both by segmental mechanisms and by commands descending from supraspinal centres. Such signals sum together or cancel out and their balance is vital for maintaining normal functions of the nociceptive system. The disruption of inhibitory input to projection neurons and/or facilitating input to inhibitory interneurons may contribute largely to development of painful syndromes as we know them (Heinricher et al. 2009).

3. NEUROPATHIC PAIN

3.1. Specifics and etiology of neuropathic pain

Neuropathic pain is defined by the International Association for the Study of Pain as chronic pain caused by a lesion or disease of the somatosensory system (IASP 2015). As such, neuropathic pain can occur independently on other tissue damage and in contrast with other kinds of pain it lacks the protective purpose. It is often irreversible and it persists even after the cause of nerve damage is eliminated (Ueda 2008).

Neuropathy is often considered as a pathological syndrome caused by a range of various nerve damaging stimuli (Jensen et al. 2011). Possible causes of neuropathic pain development include trauma, metabolic syndromes (such as diabetic polyneuropathy), drug- or toxin-induced injury (such as chemotherapy treatment of certain cancer patients), hereditary disorders, malignancy and infective or post-infective nerve damage (such as herpes or HIV infections) (Zimmermann 2001). In contrast, the clinical manifestation of nerve damage of different etiology is much unified. Typically, patients evince aberrant sensory perception with stimulus-independent persistent pain as the dominant symptom. Common disorders are allodynia (pain perception upon an innocuous tactile stimulus) and hyperalgesia (exaggerated pain sensation caused by a mildly noxious stimulus). Patients also often suffer from reduced limb sensitivity caused by the lesion (Baron et al. 2010). The counterpart of these syndromes in model animals is tactile allodynia leading to withdrawal behaviour of affected paw in reaction to a harmless stimulus.

The original damage of primary afferent nerve fibers (PAFs) causes an immediate small burst of activity, which quickly subsides. The retrograde signalling from the lesion site towards the cellular soma undergoes specific changes such as formation of retrograde signalling complexes, which consequently set off pathological processes in DRG neurons acting on the level of gene expression, post-translational modifications and protein production. Following axotomy, up to a quarter of known genes show altered expression (West et al. 2015). These changes lead to altered excitability of DRG neurons and also to their spontaneous activity, which can cause sensory disorders mentioned above and contribute to a process known as central sensitization. Accordingly this aberrant activity influences the nociceptive transduction at higher stages and provokes responses in the spinal cord, the brain stem and the cortex (West et al. 2015).

The development and sustaining of neuropathic pain is also closely associated with glial response and with local reaction of immune system. The neuronal excitability is influenced not only by spontaneous activation of peripheral fibers but also by various immune mediators released by

CNS-residing astrocytes and microglia or by infiltrating free immune cells (such as T-cells). Neuropathic pain is now therefore considered a neuroimmune disorder (Grace et al. 2014).

Although there is a high number of patients suffering from neuropathic pain – it is currently estimated to affect approximately 6 – 8% of the population (Smith and Torrance 2012) – the existing therapy is not sufficient. Up to two thirds of patients experiencing neuropathic pain cannot achieve proper pain relief, which is mostly caused by our inability to identify and target the mechanisms that trigger and help sustain pain in individual patients (Finnerup et al. 2010).

3.2. Animal models of neuropathic pain

Because of obvious ethical problems, there are limitations to the research of neuropathic pain in humans. To describe and get to understand its molecular processes, it is possible to employ studies of animal models, using most conventionally rats and mice. There are several well established models of neural injury that induce the development of neuropathic pain-like behaviour. These models can be systematically classified as peripheral or central and they are either non-traumatic or traumatic. Given the focus of this work, here we will be only considering peripheral neuropathic models from which the traumatic models will be described more closely.

Non-traumatic models of neuropathic pain cover a broad range of chemical treatments in order to simulate various pain-triggering states. These include for example neurotoxic substances and chemotherapeutic drugs (such as streptozotocin) or proteins associated with neuropathic pain states (Jaggi et al. 2011). In traumatic models, the injury is inflicted on a peripheral nerve by ligation, transection, crush or a combination of these mechanical deformations. In rats and mice, the most convenient site of surgical intervention is along the sciatic nerve (*n. ischiadicus*), which is the biggest nerve of the lumbosacral plexus formed by nerve fibers from L3, L4 and L5 spinal roots. Particular animal models differ in the level and the extent of the executed injury (Jaggi et al. 2011).

All the surgical work on animals is done under anesthesia and the development of neuropathic pain-like behaviour can be observed within several days. Treated animals show signs of spontaneous pain, such as paw guarding, excessive licking or limping, and they develop allodynia and hyperalgesia to mechanical and thermal stimuli, which are considered as markers of neuropathic pain and their measurements allow the quantification of animal pain (Jaggi et al. 2011). Autotomy (self-attack and mutilation of the enervated limb) can also occur but it is unclear whether

such behaviour reflects the painful experience or rather the excess of grooming in the absence of sensory feedback (Kauppila 1998).

The oldest model, the sciatic nerve transection (SNT), is based on complete axotomy of *n. ischiadicus*. In this case, the common sciatic nerve is exposed at the level of mid-thigh, firmly tied at two places (approx., 1cm apart) and cut in between. Following such an intervention, a neuroma develops on the proximal stump and the neuropathic pain occurs together with desensitization of the distal limb. However, the state of complete denervation is not very common, except of in amputation patients, and together with severe motor deficit it represents a major limitation of this animal model as it is impossible to test experimental animals for allodynia and hyperalgesia (Jaggi et al. 2011; Muthuraman et al. 2008).

One of the most utilized models of peripheral mononeuropathy in rats is the chronic constriction injury (CCI), developed by Bennet and Xie (Bennett and Xie 1988). The common sciatic nerve is exposed and loosely tight with 3-4 ligatures of chromic gut or silk. This way the nerve is compressed but not completely interrupted and the epineural circulation is preserved. Following the constriction, intraneural edema and focal ischemia develop at the site of injury and certain neural fibers undergo Wallerian degeneration (De Vry et al. 2004). Partial damage to the nerve induces sensitization of both A and C fibers (Gabay and Tal 2004), which leads to development of classical pain-related behavioural changes including allodynia and hyperalgesia. These signs can be observed within a week after the surgery and they persist for at least 7 weeks. Noted variability in pain-like behaviour probably corresponds to the level of tightness of the ligatures and to the type of suture material (Dowdall et al. 2005; Jaggi et al. 2011). In human medicine, the specific behaviour observed in CCI mononeuropathy model correspond to causalgia, complex regional pain syndrome and entrapment neuropathy in patients (Bennett and Xie 1988).

Other frequently employed model is partial sciatic nerve ligation (PNL/PSNL), developed by Seltzer. The common sciatic nerve is exposed and the dorsal third to half is tightly ligated with a silk suture (Seltzer et al. 1990). Quick development of behavioural alterations is observed, again within a week after the surgery, and majority of the changes last for at least 6 weeks (Dowdall et al. 2005). In relation to the human medicine, the PNL model is useful for research of causalgiform pain disorders, as these are mainly caused by partial nerve injury (Seltzer 1995).

There are more well-established peripheral models of neuropathic pain, including spinal nerve ligation (SNL), spared nerve injury (SNI) or chronic constriction of dorsal root ganglion (CCD). Apart from the sciatic nerve models, studies employing surgical interventions on other peripheral nerves are also in use (such as tibial and sural nerve transection model and ligation of the common peroneal nerve), as well as models working with various different ways of inflicting the

injury (such as sciatic cryoneurolysis, laser- and photochemically-induced sciatic nerve injury). Most of the models mentioned above have been developed in rats but have their equivalent models in mice, with minimal protocol alternation and comparable results (Jaggi et al. 2011).

4. TRPV1 CHANNEL AND ITS ROLE IN NOCICEPTIVE TRANSMISSION

4.1. TRP channels

TRP channels are tetrameric cation transporters located in plasmatic and intracellular membranes (Dong et al. 2010) that facilitate the transmembrane flux of cations and trace metal ions along their electrochemical gradients. Although TRP channels are called 'non-selective', their affinity to individual cations can be variable and most of them show some preference for Ca^{2+} ions (Nilius et al. 2005a; Ramsey et al. 2006). Activity of TRP channels leads to an accumulation of cations in the cytoplasm to depolarization of the cellular membrane. The of transmembrane voltage triggers numerous intracellular processes, such as activation of voltage-gated ion channels, and can ultimately result in the initiation of an action potential in a neuronal cell, or in the contraction in a muscle cell. However, the influx of cations, especially when talking about Ca^{2+}, is by itself considered as an important inward signal in both excitable and non-excitable cells, which puts the TRP channels in the centre of many vital cellular processes (Nilius and Owsianik 2011).

In mammals, TRP channels are expressed by both excitable and non-excitable cells within most tissues, where they play an important role in maintaining the homeostasis by regulating the intra- and extracellular cation balance, pH, and osmolality. They are in their turn sensitive to various intracellular signals, such as increased concentration of cytoplasmic Ca^{2+}, phosphatidylinositol-4,5-bisphosphate (PIP_2), or phosphorylation (Nilius and Owsianik 2011). Neuronal TRP channels in particular are implicated in registering and transmission of sensory stimuli from the external environment and distinct TRPs have been showed to participate in processing of taste, olfaction, vision, hearing, touch, thermo-sensitivity, and pain (Boonen et al. 2016; Stucky et al. 2009). Moreover, following activation, a single TRP channel is capable of transporting millions of cations into the intracellular space, where each of them interacts with multiple targets. Therefore, TRP channels in neurons also function as amplifiers of the transmitted signal (den Dekker et al. 2001).

The TRP family consists of 7 sub-families: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPN (NOMPC-like), TRPP (polycystin), and TRPML (mucolipin) (Ramsey, 2006). In mammals, 28 individually coded TRP units were so far identified that include members from all sub-families except the TRPN, since the *nompC* gene responsible for forming of TRPN units has been only found in invertebrates (Walker et al. 2000) and fish (Sidi et al. 2003). In one species, the homology between two members of the same sub-family is around 35% but it can reach up to 80% in units that originate from sequential duplicates, such as TRPC6 and TRPC7 or TRPV4 and TRPV5 (Nilius and Owsianik 2011).

Although the length and composition of TRP units vary between distinct TRP sub-families and even their members, several structural features are shared across the whole family (see Figure 4.1). A typical TRP protein spans 6 times through the cellular membrane (segments S1 - S6), leaving the carboxyl- (C-) and amino- (N-) terminals at the cytoplasmic side and forming a reentrant hydrophobic pore loop between S5 and S6 (Gaudet 2008b). The C- and N- terminals carry specific motifs and domains that participate in the cation transport itself or have other specialized functions. These include the C-terminal TRP domain that is present in all TRP channels except of TRPA1 and TRPP (Ramsey et al. 2006), the coiled-coil motif implicated in TRP polymerization (García-Sanz et al. 2004; Tsuruda et al. 2006), and a number of specific binding sites (such as the calmodulin binding site, phosphorylation sites, lipid-interaction domains…) (Zheng 2013). Interestingly, some domains have been showed to have enzymatic function – for example the Nudix hydrolase domain of TRPM2 have been reported to have an ADP-ribose pyro phosphatase activity (Tóth et al. 2014), and an atypical α-kinase domain was found at the C-terminal of TRPM6/M7 (Runnels et al. 2001).

One of the most conserved features across all TRP channels is the presence of an Ankyrin repeat domain (ARD) at the N-terminal. Ankyrin (ANK) is a 33 AA long motif in form of 2 antiparallel α-helices connected by a β-hairpin. The number of ANKs the domain is different in distinct TRPs: 3 - 4 in TRPCs, 6 in TRPVs, 14 - 15 in TRPAs and around 29 in TRPNs (Gaudet 2008a). Functionally, the ARDs have been linked to numerous processes including ion transport, cell signalling, cytoskeleton interactions, and inflammation (Gaudet 2008a; Mosavi et al. 2004). It was also suggested that ARDs might play a role in TRP polymerization and in interactions between the TRP channel and other ligands or proteins (Gaudet 2008a; Huynh et al. 2014; Jin et al. 2006).

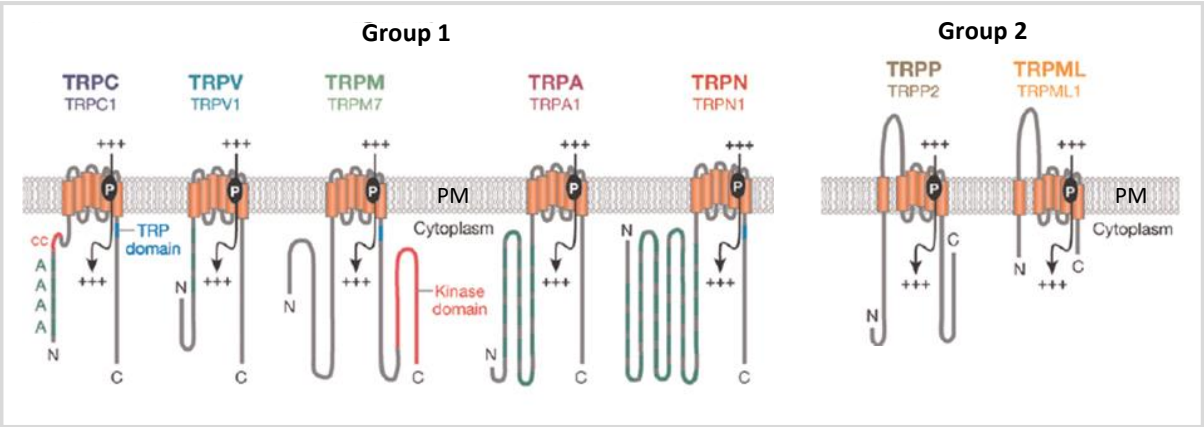

Figure 4.1: Schematic representation of TRP channels with 7 TRP sub-families divided into 2 groups. Members of first group (TRPC, V, M, A and N) are missing a large extracellular loop between S1 and S2 that is present in members of the second group (TRPP, ML). PM – plasmatic membrane; A – ankyrin domain; cc – coiled-coil domain; P – hydrophobic pore loop allowing the entry of cations (+++) (adapted from Venkatachalam and Montell 2007)

The TRP channel itself is formed by four TRP units surrounding the cationic pore, more often as a homo-tetramer, although there is ample evidence of hetero-tetramers as well (Hofmann et al. 2002; Venkatachalam and Montell 2007). These hetero-tetramers were mostly observed in cultured cells with co-transfected genes for distinct TRP units so their viability *in vivo* requires further research, it is however well recognized that such combination of sub-units can create a vast range of potential TRP channel properties (Cheng et al. 2010). Most of the known hetero-tetramers are formed by members of the same TRP sub-family, such as TRPC1 and TRPC5/C5 (Strubing et al. 2001),or TRPM6 and TRPM7 (Chubanov et al. 2004). A functional hetero-tetramer can be also formed by members of different TRP sub-families. The TRPC1 unit is considered as the most versatile, as it was showed to form tetramers with TRPP2 (Kobori et al. 2009) as well as with TRPV4 (Liu et al. 2005) and TRPV6 (Schindl et al. 2012). In addition, there are several proofs of heteromerization outside of the TRP family - for instance, the TRPP2 subunits are capable to form a trimer, which then forms a proper channel by interaction with a PKD1 subunit (a 11 putative transmembrane segment-containing protein) (Yu et al. 2009).

Until recently, the general structure of the TRP tetramer had been only visualized with low resolution, complemented by more detailed crystallography images of few domains (e.g. ARDs). Based on sequence comparisons and the available data, the TRP structure was assumed to resemble that of a voltage-gated potassium channel. More recently, a detailed image of a tetramer of the most researched TRP member, TRPV1, was obtained using the cryo-EM at a resolution of 3.3 A° (Cao et al. 2013, Liao et al. 2013) and the channel was also visualized in the natural bilayer environment using a combination of cryo-EM with lipid nanodisc technology as it is showed in Figure 4.2 (Gao et al. 2016). A detailed structural image of TRPA1 is also already available (Paulsen et al. 2015).

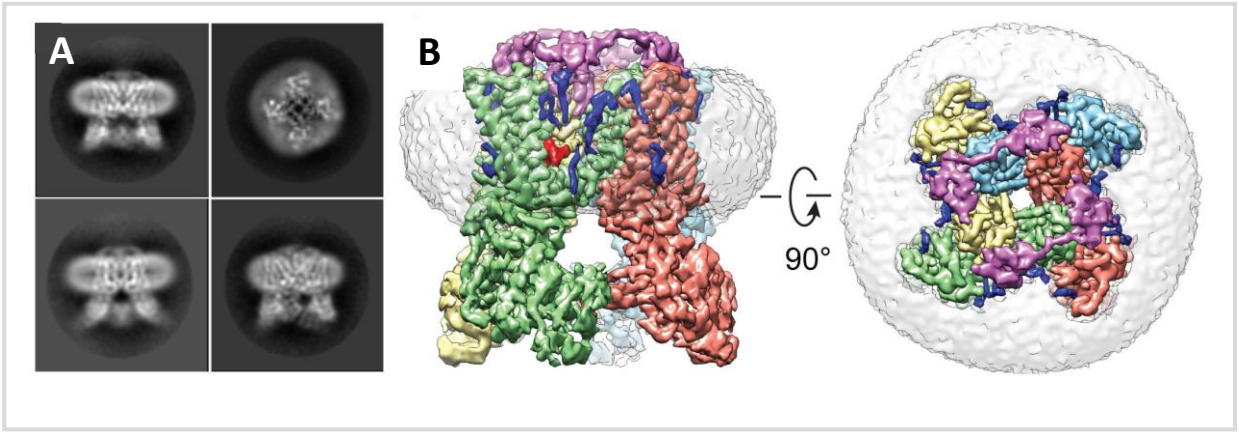

Figure 4.2: Molecular structure of TRPV1 tetramer showed when incorporated in a membrane nanodisc. (**A**) Side and top views of reference-free 2D class averages. (**B**) Side and top views of 3D reconstruction of TRPV1-ligand-nanodisc complex with color-coded monomers. Two molecules of DkTx toxin (purple) are showed atop the channel and a molecule of RTX (red) in the vanilloid binding pocket. (adapted from Gao, et al. 2016)

4.2. TRPV1, the capsaicin receptor

4.2.1. TRPV sub-family

In mammals, the TRPV family comprises 6 members described up to this date that can be separated in two distinct groups according to their cation selectivity (even though this categorization doesn't follow the TRPV genetic tree). Constituting the first group, TRPV1 – 4 represent non-selective cation channels (with permeability ratio P_{Ca}/P_{Na} between 1 and 10) that are sensitive to diverse stimuli including many messenger molecules, extreme temperatures and chemical and/or mechanical stress (Clapham 2003; Nilius et al. 2008). The second group of the TRPV subfamily gathers TRPV5 and TRPV6, which show high selectivity for Ca^{2+} (with P_{Ca}/P_{Na} >100) that is unique amongst all TRP channels. Such selectivity allows these channels to participate in Ca^{2+} absorption and reabsorption across tubule epithelia in intestines and kidney (den Dekker et al. 2003).

Like TRPV1, which will be discussed in more detail later, all other members of the first group have been implicated in thermosensitivity and nociception (Premkumar and Abooj 2013). TRPV2, abundant in neuronal cells, is generally considered as heat sensitive with a high activation threshold (>52 °C). This was originally deduced based on data from monkey and mice (Tominaga and Caterina 2004) and consequently confirmed in rat/mouse TRPV2 expressed by HEK293 cells. However, when human hTRPV2 was used, no heat sensitivity was observed and hTRPV2 didn't show any response to some of the established mouse TRPV2 agonists (Neeper et al. 2007). Other TRPV member, TRPV3, is mostly expressed in keratinocytes, cells surrounding hair follicles, and in humans (although not in mice) also in neurons of DRG, spinal cord and brain (Huang et al. 2011). Human TRPV3 is activated by physiological temperature within range of 32 to 39 °C and it was implicated in development of hypersensitivity, as its levels are augmented in nociceptive DRG neurons following a nerve injury (Facer et al. 2007; Nilius et al. 2014). Lastly, TRPV4 is a polymodal receptor that is expressed in a large variety of human tissues and responds to diverse mechanical and chemical stimuli. It is also sensitive to mild temperatures between 24–32 °C, suggesting its constitutive activity in humans (Nilius et al. 2003). However, although both TRPV3 and TRPV4 respond to thermal stimuli, thermal perception isn't probably their main function in the organism, since this ability is not altered in TRPV3-/TRPV4- knock-out mice (Huang et al. 2011).

4.2.2. Structural aspects of TRPV1

The transient receptor potential vanilloid 1 (TRPV1) is the first identified member of the TRPV sub-family, whose expression and function became subject of many studies since its first

cloning. Its existence was discovered as a result of an extensive search for a molecular target of capsaicin (CAPS), the pungent ingredient in chili peppers, which exposure *in vivo* elicits burning sensation and pain. Capsaicin had been known for its ability to excite nociceptive neurons and to make them release inflammatory mediators (Szallasi and Blumberg 1999). So when in 1997 Caterina et al. isolated the receptor using a Ca^{2+} imaging-based expression cloning method, they named it vanilloid receptor subtype 1 (VR1) according to the vanilloid effective moiety of capsaicin (Caterina et al. 1997). Following patch-clamp studies then confirmed that the application of CAPS to membrane patches from TRPV1-expressing HEK293 cells evokes single-channel openings independent on cytosolic second messengers (Caterina and Julius 2001; Tominaga et al. 1998).

The human *trpv1* gene is located on the short arm of chromosome 17 and is approx. 44 kbp long. Until now, three distinct splice variants have been described (VR.5'sv, TRPV1β and TRPV1var), that have been showed to influence functional properties of TRPV1 receptor depending on their specific representation (Schumacher and Eilers 2010). The nucleotide sequence of the *trpv1* gene is highly conserved amongst species – for example the homology between human and rat *trpv1* cDNA is 86%. The most rigid sequences encode transmembrane helixes, while the most variety is found in sequences that encode the termini of the receptor (Hayes et al. 2000). The final protein of a TRPV1 unit is 838 AA long (95 kDa) and folds into the classic TRP structure (Lishko et al. 2007; Moiseenkova-Bell et al. 2008; see Figure 4.3A,B).

The transmembrane segment of a TRPV unit is formed by 6 helices (S1 – S6) that are divided in two functionally separate modules. S5 and S6 together with the interconnecting outward loop create the inner pore wall, whereas S1 – S4 represent a voltage-sensitive module embedded in the membrane (Liao et al. 2013). The transmembrane segment harbors two distinct binding sites that together form a dual-gate system, in which activation of each binding site leads to a specific shift in molecular conformation (see Figure 4.3C). The lower gate (G2) is regulated by the CAPS binding site that also respond to resiniferatoxin (RTX), whereas the upper gate (G1) can be controlled by protons or by toxins, such as the double-knot toxin (DkTx) from spiders (Henderson 2013; Siemens et al. 2006). The inner pore has several restriction points with narrow inactive diameter that widen upon activation, when both gates open and allow cations pass (Liao et al. 2013).

The N-terminus is formed by 6 ANK domains that are connected to S1 with a conserved linker region. Both segments were showed to be essential for bonding with neighbouring sub-units within the tetramer, with 2 ANKs from one unit binding a β-sheet from the linker and a β-strand from the C-terminal region of another unit (Liao et al. 2013). In addition, crystallographic studies of the ARD revealed a concave surface that participates in other protein-protein interactions (Lishko et al. 2007), and distinct binding sites for calmodulin (CaM) and ATP. Both ligands modulate the

channel activity - CaM acts as an inhibitor, while binding of ATP leads to stabilization of the ARD and to regulation of Ca^{2+}/CaM-dependent desensitization (Phelps et al. 2010).

The C-terminus contains the conserved TRP domain located close to S6, which participates on tetramer assembly (Liao et al. 2013) and plays an important role in allosteric channel activation mediated through its central residues (Gregorio-Teruel et al. 2015). Apart from the TRP domain, the C-terminus carries various binding sites for intracellular ligands. These include another CaM binding site (Numazaki et al. 2003), a phosphatidylinositol bis-phosphate (PIP2) binding site (Ufret-Vincenty et al. 2011), and several sites for kinases and phosphatases (Liao et al. 2013). It has been showed that following deletion of the C-terminus, TRPV1 channel loses its sensitivity to chemical, thermal, or voltage stimuli (Vlachová et al. 2003).

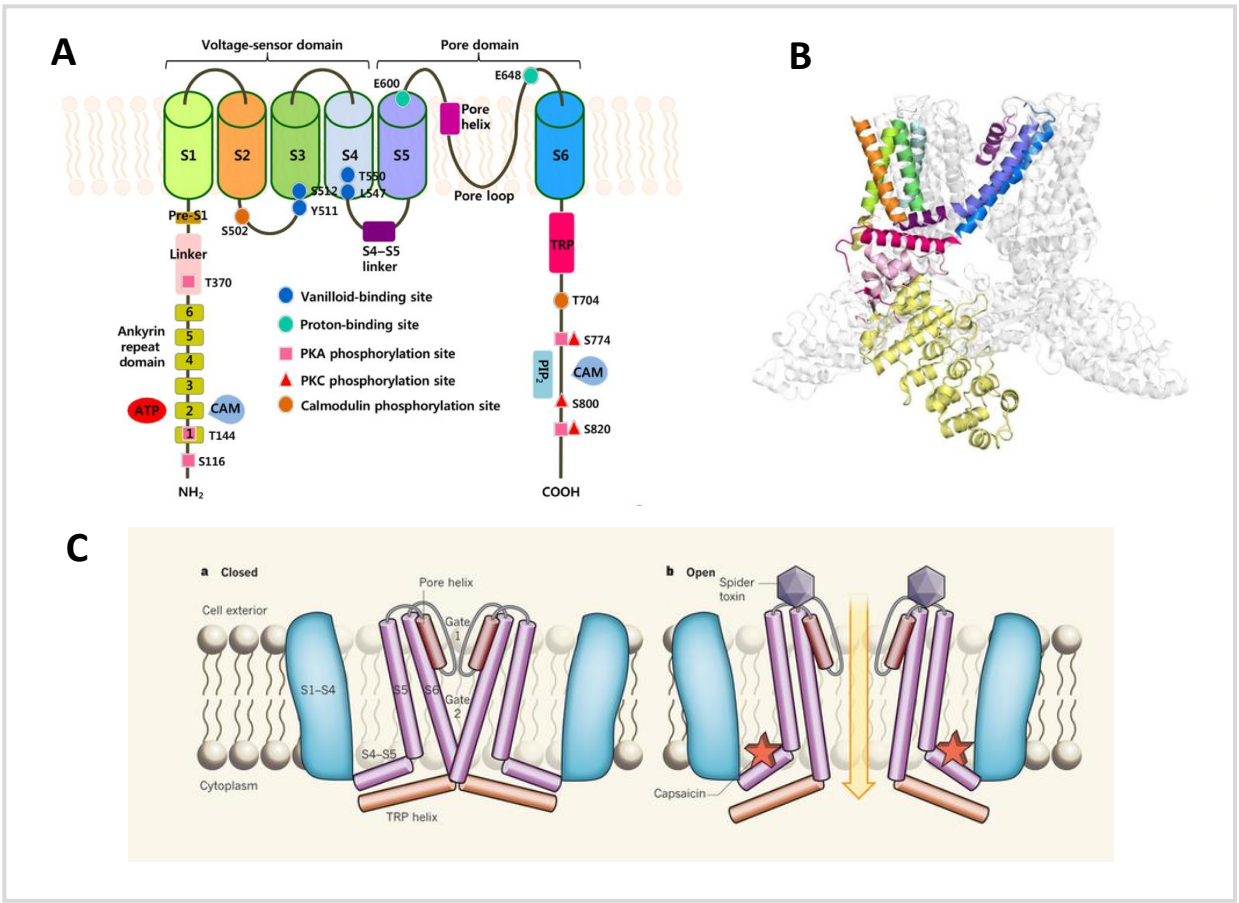

Figure 4.3: General architecture and functioning of TRPV1 channel. (**A**) Domain organization of TRPV1 is showed in a 2D view depicting all main structural TRPV1 modules including ARD, linker domain, pre-S1 helix, transmembrane domain (S1–S6), S4–S5 linker, pore helix, pore loop and TRP domain. Residues involved in the ligand-, calmodulin-, PIP2-, and ATP-binding and phosphorylation are labelled with relevant symbols. (adapted from Cui et al. 2016). (**B**) Ribbon representation of TRPV1 tetramer with of its subunits color-coded according to 4.3A (adapted from Cui et al. 2016) (**C**) The cartoon shows TRPV1 in closed (a) and open (b) states (only 2 of 4 sub-units are visible). Helices of one sub-unit are labelled, as well as CAPS and DkTx molecules depicted as bound at the open channel (a). The arrow indicates the Ca^{2+} passage through the channel (adapted from Henderson et al. 2013).

Like other TRP channels, TRPV1 units assemble into a tetramer to form the cation channel, which is composed of a dense trans-membrane segment and a large cage-like cytosolic structure made of N- and C-termini of all sub-units (Cao et al. 2013; see Figure 4.3B). Although homo-tetramers are more frequent, it commonly forms hetero-tetramers with other members of TRPV family (Liapi and Wood 2005). In fact, as it was documented using spectra-FRET measurements, all thermo-sensitive TRPV channels are capable of forming hetero-tetramers amongst themselves and these mixed channels can exhibit extraordinary conductive and gating properties (Cheng et al. 2007). Also it appears that TRPV1 can co-assemble with TRPA1, a member of different TRP sub-family with whom TRPV1 frequently co-localizes in sensory neurons (Staruschenko et al. 2010).

4.2.3. TRPV1 activation

TRPV1 is a non-selective cation channel with considerable preference for Ca^{2+} ions - its permeability ratio for calcium is $P_{Ca}/P_{Na} = 9.6 / 1$ and for magnesium $P_{Ca}/P_{Mg} = 5 / 1$, measured during the activated state (Caterina and Julius 2001). Apart from cations, TRPV1 is also permeable for polyamines putrescine, spermidine and spermin (Ahern et al. 2006), for organic cationic dyes (Meyers et al. 2003), and for aminoglycoside antibiotics (Myrdal and Steyger 2005). Upon its activation, TRPV1 opens its gating system and allows cations enter the intracellular space, it has therefore an outwardly-rectifying current-voltage relationship (Caterina and Julius 2001). Its gating has a Closed-Open-Closed character with mean burst duration around 54ms, and this time can decrease if TRPV1 forms a hetero-tetramer with other TRPV units (Vennekens et al. 2008).

An important feature of the TRPV1 channel is its ability to respond to stimuli of diverse modalities – it's a polymodal receptor that can function as molecular stimulus integrator, especially in primary afferent fibers (Caterina and Julius 2001). It is known that TRPV1 can be activated by high temperatures > 43 °C (Tominaga and Caterina 2004) but the mechanism of the stimulus transduction is still unclear, only with suspected involvement of the C-terminus and the outer pore region of the channel (Devesa et al. 2011). TRPV1 is also activated by low pH < 6 (Davis et al. 2000) and by a broad variety of ligands (described in more detail in this chapter) that interact with numerous binding sites of the receptor (see Figure 4.3A). In addition, TRPV1 is known to respond through its voltage-sensor domain to non-physiological high membrane potential (Yao et al. 2011).

The temperature and voltage sensitivity of TRPV1 have been closely linked, when it was showed that voltage-dependence of the channel shifts substantially with higher temperatures. While at room temperature the threshold for voltage-dependent activation is around +150 mV and therefore out of reach within a living cell, rising temperature induces gradual lowering of the

threshold that reaches approx. -50 mV between 40 and 45 °C (Voets et al. 2004) and thus sensitized TRPV1 becomes a voltage-gated cation channel (Nilius et al. 2005b; Szolcsányi and Sándor 2012).

The pH sensitivity of TRPV1 channels represents in fact another form of ligand activation, as it is based on interactions between protons and specific proton-binding sites in the extracellular region of the channel (Kweon et al. 2015). The character of TRPV1 response to low pH depends on the acidity intensity. It was showed that in strongly acidic pH the channel directly activates, whereas milder pH rather causes its sensitization (Aneiros et al. 2011). During tissue acidosis, the temperature activation threshold moves towards the normal body temperature, which might be responsible for TRPV1 activation during ischemia or inflammation (Vennekens et al. 2008).

Unlike many other members of the TRP family, TRPV1 receptor is activated by a broad variety of ligand molecules, of which many had been well known for their irritating effects in mammals even before the cloning of TRPV1 itself. The most archetypal TRPV1 agonist is capsaicin, an alkaloid by-product of the vanillin metabolic pathway in plants of the genus *Capsicum* (chili peppers). Its application elicits painful burning sensation in any contacted tissue due to its binding to TRPV1 receptors in nociceptive neurons (Szallasi and Blumberg 1999, Caterina et al. 2000), and these effects are mediated exclusively through TRPV1, as was showed in capsaicin-insensitive *Trpv1* knock-out mice (Caterina et al. 2000). CAPS, like other cognate substances, contains a vanilyl domain with an aromatic circle, which is necessary for binding to TRPV1 receptor. Thanks to its lipophilic nature, CAPS easily passes through the plasmatic membrane and interacts with the TRPV1 vanilloid pocket, which is a cavity formed by S4 and S5 transmembrane domains in the intracellular region of the channel (Yang et al. 2015).

Upon the CAPS-mediated TRPV1 activation in peripheral receptors, the hyperactivity of PAFs manifests as acute pain and leads to temporary lowering of thermal and chemical activation threshold (sensitization) of these fibers. This excitatory phase is followed by a refractory phase (desensitization) that can have variable duration depending on the character of the activation (Caterina and Julius 2001, Szallasi and Blumberg 1999). Acute desensitization, which usually lasts for several hours, occurs approx. 20 s after the beginning of acute CAPS application and it is assumed that it protects the cell from Ca^{2+} overdose (Touska et al. 2011). During acute desensitization, the receptor cell doesn't respond to normally effective thermal stimuli, but it remains sensitive to chemical ligands (Williams and Zieglagänsberger 1982). On the other hand, repeated CAPS treatment results in long-term desensitization, which can last for many days (Caterina and Julius 2001). Moreover, following prolonged application of highly concentrated CAPS, affected nerve endings and neurons undergo so-called neurotoxic inactivation/degeneration that leads to a dose-dependent loss of TRPV1 expressing C and Aδ PAFs (Nagy and Van Der Kooy

1983) and consequent nearly complete local pain insensitivity. CAPS treatment has therefore broad utility both in research and in clinical pain management.

CAPS is the most common member of a group of related molecules called capsaicinoids and it has also several natural analogs produced by various plants, such as resiniferatoxin (RTX), eugenol, guaiacol, gingerol, or zingeron (Szallasi and Blumberg 1999). The most important of these substances is probably RTX, a terpene found in the latex of several members of the genus *Euphorbia,* which was reported to be 3–4 orders of magnitude more potent than CAPS (Szallasi and Blumberg 1989). Its molecule is larger, more lipophilic and has 100 – 1000 times higher affinity for TRPV1, which is the reason why it is often used as experimental TRPV1 activator (Appendino and Szallasi 1997). Interestingly, the perceived effects of RTX are different from the ones caused by CAPS, as the acute application of RTX skips the excitatory phase and doesn't elicit pain, but leads to a long-lasting dose-dependent desensitization (Avelino et al. 1999). In general, it appears that the pungency of TRPV1 agonists does not depend so much on their potency but rather on their lipophilicity. Highly lipophilic agonists are less pungent, because they activate TRPV1 more slowly, which results in delaying or even suppressing the generation of an action potentials in sensory neurons (Ursu et al. 2010).

Apart from vanilloids, many other naturally occurring exogenous TRPV1 agonist were identified amongst plant and animal irritants, such as piperine from black pepper (McNamara et al. 2005), allicin from onion and garlic (Salazar et al. 2008), or peptide spider toxins called vanillotoxins (VaTx1, 2, and 3) from the tarantula *Psalmopoeus cambridgei* (Siemens et al. 2006). Recently, a special attention was paid to a similar toxin from the Earth Tiger tarantula that was showed to selectively, very strongly and irreversibly bind to the upper gate at the outer side of the TRPV1 channel and thus cause its permanent opening (Bohlen et al. 2010). The structure of this spider toxin, which was named double knot toxin (DkTx), is very particular, with two symmetrical inhibitor cysteine knot (ICK) domains (also found in vanillotoxins) interacting simultaneously with two TRPV1 sub-units (Bohlen et al. 2010). Using DxTx for locking on TRPV1, it was possible to finally visualize the channel in its open position (Liao et al. 2013; see Figure 4.2B).

Endogenous agonists of TRPV1, known as endovanilloids, form a diversified group that can be roughly divided into lipid and non-lipid TRPV1 agonists. The first discovered endovanilloid was anandamide (AEA), a fatty acid neurotransmitter derived from arachidonic acid that interacts with TRPV1 on perivascular sensory nerves and through its activation induce vasodilatation. AEA represents a link between TRPV1 and the cannabinoid system, because it binds cannabinoid receptors that are also abundant in neurons of sensory pathways (Zygmunt et al. 1999a). Since AEA, many other endovanilloids were found that are products of arachidonic acid metabolism, such as

leukotriene B4, prostaglandins, lipoxygenase products 5-, 12-, and 15-hydroperoxyeicosatetraenoic acids (5S-, 12S-, 15S-HPETE) (Huang et al. 2002; Hwang et al. 2000) and various conjugates of biogenic amines including N-arachidonoyl-dopamine (NADA, also an endocannabinoid), N-oleoyl-ethanolamine (OLEA), and N-oleoyl-dopamine (OLDA) (Almási et al. 2008).

N-oleoyl-dopamine (OLDA), which was used for TRPV1 activation in the practical part of this work, was confirmed as a selective TRPV1 agonist that shows more potency than most other known endovanilloids. It was reported that OLDA is capable to induce TRPV1-mediated dose-dependent thermal hyperalgesia following an intraplantar injection, which proves the important role of peripheral TRPV1 in nociceptive transmission (Chu et al. 2003). Using patch-clamp recordings, a significant effect of OLDA was also reported at the spinal cord level, where its acute application led to an increase in frequency of miniature postsynaptic excitatory currents (mEPSC) measured in secondary sensory neurons in the spinal cord dorsal horn, and this response could be blocked by a pre-treatment with TRPV1 antagonists (Spicarova and Palecek 2009). While in neurons from healthy animals it was necessary to administer highly concentrated OLDA (10 μM) to obtain the response, a much lower concentration of OLDA (0.2 μM) was needed to achieve the same effect during peripheral inflammation (Spicarova and Palecek 2009). Low concentration of OLDA was also effective when applied together with an inflammatory cytokine tumor necrosis factor α (TNFα) (Spicarova and Palecek 2010). Based on these findings, it was proposed that TRPV1 activation by physiologic low-concentration of endovanilloids might significantly affect spinal nociceptive transmission during pathological states when TRPV1 receptors are sensitized.

4.2.4. TRPV1 distribution in the organism

With the help of reverse transcription-polymerase chain reaction (RT-PCR) it was showed that TRPV1 is expressed by a broad variety of tissues throughout the human body, with the highest reported abundance in the dorsal root ganglion (DRG), nodose ganglion and trigeminal ganglion (TG) (Pingle et al. 2007). Lower level of TRPV1 expression was detected in the spinal cord and various brain regions (Fernandes et al. 2012) and its presence was also found in non-neural tissues in kidney, pancreas, gonads, spleen, stomach, small intestine, lung and liver (Hayes et al. 2000).

In rats, a variety of techniques were used to localize TRPV1, such as RT-PCR, immunohistochemistry, *in situ* hybridization, or northern blotting. Similarly to humans, TRPV1 was identified in tissue of the DRG (Hayes et al. 2000), trigeminal ganglion (Caterina et al. 1997) and numerous areas in the central nervous system (Mezey et al. 2000). Apart from the neuronal tissue, TRPV1 was found in kidney, spleen, placenta (Hayes et al. 2000), and bladder epithelia

(Birder et al. 2001). The distribution of TRPV1 in mice is similar to rat (Cristino et al. 2006; Mishra et al. 2011) with additional discovery of TRPV1 expression in smooth muscle cells in small arteries of thermoregulatory tissues, where its activation leads to vasoconstriction (Cavanaugh et al. 2011b).

Amongst neurons residing in sensory ganglions (DRG, TG), TRPV1 is found in approx. 35–50 % of cells with small diameter that represent cellular bodies of non-myelinated (C) and lightly myelinated (Aδ) PAFs (Kobayashi et al. 2005). These TRPV1-positive fibers include subsets of peptidergic and (to lesser extent) non-peptidergic neurons implicated in temperature sensitivity and nociception (Ferrandiz-Huertas et al. 2014). In their peripheral endings located in the target tissue, TRPV1 receptors are found in profusion and are known to act as transducers and molecular integrators of peripheral stimuli. TRPV1 is also found in central terminals of PAFs that form synapses with secondary sensory neurons in the spinal cord (Brederson et al. 2013; Valtschanoff et al. 2001) and it has been documented in mice that distinct populations of TRPV1+ and TRPV1- DRG neurons target different areas in the dorsal horn of the spinal cord (Cavanaugh et al. 2011a). Using Cre-induced lineage tracing, the same study also showed that the expression of TRPV1 across the population of DRG neurons change in the course of the ontological development. While in young animals TRPV1 was detected in a great number of neurons, in adult mice the expression of TRPV1 was restricted to smaller subset of mostly peptidergic neurons (Cavanaugh et al. 2011a).

At the spinal level, studies using radioactively labeled TRPV1 agonist RTX ([^{3}H]RTX) revealed abundant presence of TRPV1 receptors in the dorsal horn (DH) of the spinal cord in human, pig and rat. Different levels of TRPV1 were measured in distinct segments of the spinal cord, the lumbar segment showing about doubled reactivity in comparison to the thoracic or cervical part (Szallasi et al. 1995). At the cross-section, the highest TRPV1 reactivity was observed in superficial laminae I and II, where the first synapses of the nociceptive pathway are located (Guo et al. 1999; Szallasi et al. 1995). Also with the help of [^{3}H]RTX, Valtschanoff et al. showed that the TRPV1 expression is detected both presynaptically in central PAF terminals and postsynaptically in spinal secondary sensory neurons (Valtschanoff et al. 2001). The presence of TRPV1 in DH neurons was later confirmed through extensive histological and biochemical analysis in a cell culture model of the spinal cord with degenerated PAFs (Ferrini et al. 2010). With the help of immunohistochemical labeling, co-localization of TRPV1 and a vesicular glutamate transporter 2 (vGluT-2) was reported in DH neurons, suggesting their excitatory nature (Zhou et al. 2009), yet another study found TRPV1 expression in GABAergic inhibitory interneurons (Kim et al. 2012).

However, following the destruction of TRPV1-positive PAFs by rhizotomy or sub-maximal doses of CAPS, it is no longer possible to detect any TRPV1 expression in DH neurons (Roberts et al. 2004; Valtschanoff et al. 2001). These findings are supported by results of a study using *Trpv1*

knock-in mice that confirmed TRPV1 presence only in the central branches of DRG neurons (Cavanaugh et al. 2011b). This evidence indicates that (a) most of the TRPV1 that had been detected in the DH were indeed located within central terminals of PAFs, and/or that (b) the expression of TRPV1 in secondary DH neurons is dependent on afferent input from the periphery.

It is necessary to state that in humans, as well as in animal models, the TRPV1 reported in various non-neural tissues is found not only in nerve fibers innervating the organs in question, but also in local epithelial cells, glial cells and in cells of immune system (Nilius and Owsianik 2011). In case of the periphery and the spinal cord, these non-neuronal cells are more and more implicated in inflammatory and neuropathic pain transmission and their function is believed to be at least partially mediated by TRPV1.

4.3. Spinal TRPV1 in normal and pathological nociception

4.3.1. TRPV1 signalling in the dorsal horn of the spinal cord

As was said in the previous chapter, TRPV1 receptors are abundantly expressed in bodies, peripheral endings and central terminals of C and Aδ PAFs that mediate nociceptive and thermal sensory transmission. While the pivotal role, which TRPV1 plays in the periphery as a transducer of thermal and chemical stimuli into nociceptive signalling, is unquestionable, its function at the first synapse of is less clear. Since TRPV1 expression in secondary sensory neurons in the DH is still being discussed, for the purposes of this work I have assumed that most of the effects observed in the DH after the application of TRPV1 agonists are mediated through presynaptic receptors, although the participation of postsynaptic TRPV1 has not been ruled out (Spicarova et al. 2014b).

The population of TRPV1-expressing nociceptors mediates selectively noxious thermal stimuli, as was showed by behavioural testing of rats with chemically-induced ablation of TRPV1+ PAFs. These animals suffered almost complete loss of noxious heat sensitivity but their mechanical sensitivity was maintained, in contrst to the animals with genetic ablation of PAFs expressing the MrgprD receptor (approx. 90 % of cutaneous non-peptidergic C fiber nociceptors) that showed impaired mechanical sensitivity with intact thermal sensitivity (Cavanaugh et al. 2009). The thermal selectivity was also confirmed by extracellular patch-clamp recordings from lumbar DH neurons after the inactivation of TRPV1+ afferents with intrathecally injected CAPS, which showed diminished response of secondary neurons to noxious heat stimuli while the response to noxious mechanical stimulation was unchanged (Zhang et al. 2013). Using a high-dose intrathecal application of RTX, it is possible to destroy central terminals expressing TRPV1 and at the same

time preserve cellular bodies and peripheral receptors of the nociceptors. Such local inactivation, however, had no significant effect on perception of noxious heat (Bishnoi et al. 2011), whereas a RTX-induced systemic ablation of TRPV1+ afferents resulted in thermal analgesia lasting for several months (Bishnoi et al. 2011; Jeffry et al. 2009).

Amongst DH sensory neurons, only about 10% are neurons projecting to higher centres, while the rest comprises of local interneurons. In lamina II, the projecting neurons are even sparser, virtually absent. TRPV1+ PAFs form connections both with projection neurons and interneurons that in their turn contact other interneurons and projection neurons and modulate their output (Ribeiro-da-Silva and De Koninck 2008; Todd 2015). The majority of TRPV1+ afferents form synapses in laminae I and II. In lamina I, most of all neurons respond to electrical stimulation of C fibers and a subset of neurons react to stimulation of Aδ fibers, however there are no reliable data concerning directly TRPV1+ synapses (Ikeda et al. 2003; Lu and Perl 2005). In lamina II, 30% to 88% of neurons responded to capsaicin application by increased mEPSC frequency (Nakatsuka et al. 2002; Petitjean et al. 2012). Moreover, many lamina II glutamatergic interneurons were observed to form synapses with lamina I projection neurons and it is believed that these internal circuits play a central role in the integration of nociceptive information (Cordero-Erausquin et al. 2016). Conversely, no reaction to CAPS was observed in neurons of deeper laminae III and IV (Schneider 1992), with the exception of a subset of neurons expressing receptors for SP that presumably contact TRPV1+ terminals through dendritic spines extended into lamina II (Petitjean et al. 2012).

Central endings of TRPV1+ afferents produce glutamate as the main neurotransmitter, together with other neuroactive substances such as calcitonin gene related protein (CGRP) and substance P (SP) (Cavanaugh et al. 2011a). Postsynaptic neurons contacted by these afferents primarily express on their surface glutamate receptors, although TRPV1+ afferents also contact population of neurons that express the receptor for SP, neurokinin-1 receptor (NK_1R). Multiple fluorescent staining showed that amongst DH neurons, NK_1R co-localize mostly with NMDA receptors and much less with AMPA receptors (Hwang et al. 2004). Following acute application of CAPS, an increase in mEPSC frequency was observed in neurons in laminae I and III/IV that represented 73% and 43% respectively of local neurons expressing NK_1R (Labrakakis and MacDermott 2003). Using a combination of immunohistochemical staining and retrograde tracking, Hwang et al. showed that these NK_1R-positive projecting neurons belong to a monosynaptic sensory pathway going from the spinal cord to the lateral parabrachial nucleus in the brainstem (Hwang et al. 2003). Later, it was proven with the help of the same techniques supported by patch-clamp recordings that the parabrachial nucleus participates in the transmission of short-latency nociceptive information to dopaminergic neurons (Coizet et al. 2010).

Upon the activation of TRPV1 receptors at peripheral nociceptive endings, central terminals located in the superficial DH release glutamate and other neurotransmitters into the synapse (Ueda et al. 1993; Ueda et al. 1994). Similar effect was observed after direct stimulation of spinal TRPV1 with the help of patch-clamp recordings from DH neurons, when the acute application of various TRPV1 agonists led to a dose-dependent increase in spontaneous glutamate release, measured as a change of frequency of miniature or spontaneous excitatory postsynaptic currents (mEPSC, sEPSC) (Jeffry et al. 2009, Spicarova and Palecek 2009). This increase in synaptic activity was blocked by TRPV1 antagonist capsazepine and a complete synaptic inhibition was achieved by combining capsazepine and CNQX, a non-competitive antagonist of AMPA and kainite glutamate receptors (Yang et al. 1998). The same results were obtained from young animals (rats within P0 – P10), with a significantly bigger CAPS-induced increase of EPSC frequency in older animals (P9 –P10) (Baccei et al. 2003). Following treatment with inhibitors of glutamate receptors (kynurenic acid – KYNA; 5-amino-5-phosphonovaleric acid – AP5), secondary sensory neurons in superficial DH laminae showed a decrease but not a complete block of activity after an application of CAPS, and the residual response is believed to be sustained by SP and CGRP released from nociceptive C fibers. On the contrary, in deeper neurons from laminae III and IV, the treatment with inhibitors led to a complete blockade of CAPS-evoked synaptic transmission (Urban and Dray 1992).

Originally, presynaptic spinal TRPV1 receptors were assumed to show tonic activity only upon sensitization by phosphorylation (see Chapter 4.3.3.), as it occurs during pathological states but not under normal conditions (Lappin et al. 2006; Spicarova and Palecek 2008). However, recently Park et al. reported moderate tonic activity of TRPV1 in control mice using patch-clamp recordings from lamina II neurons. They saw a decrease in sEPSC frequency after acute application of TRPV1 antagonists and these findings were further supported by data from TRPV1 knock-out mice that also displayed reduced sEPSC frequency in lamina II neurons (Park et al. 2011).

At the spinal cord level just like in the periphery, TRPV1 receptor was showed to undergo a significant desensitization during repeated stimulation. Following the second application of CAPS (1 μM) tu a slice of the lumbar spinal cord, the response reached only 20% of the response after the first application (Ueda et al. 1993). The desensitization of TRPV1 is regulated by several mechanisms, most important of them being the Ca^{2+}-dependent signalization. It was showed in DRG neurons of neonatal rats that in presence of Ca^{2+} the kinase CaMKII phosphorylates TRPV1, whereas the phosphatase calcineurin dephosphorylates it and causes TRPV1 desensitization. The kinetics of this process are highly dependent on the level of the intracellular Ca^{2+} and on the affinity of involved enzymes for calmodulin and Ca^{2+} (Gordon-Shaag et al. 2008; Jung et al. 2004; Por et al. 2010). Apart from the Ca^{2+}system, it was reported that TRPV1 receptors can be also desensitized

by phosphatidylinositol 4,5-bisphosphate (PIP_2) (Gordon-Shaag et al. 2008). Sensitization and desensitization of TRPV1 by various modulators are described in more detail in Chapter 4.3.3.

To the general surprise, the effect that TRPV1 agonists have on sEPSC/mEPSC frequency was not observed with evoked EPSCs (eEPSCs), and moreover, the actual documented effect varied significantly between distinct PAF populations. Following acute CAPS application (1 μM), electrical stimulation of C fibers failed to elicit eEPSCs in 79% of recorded neurons, whereas after stimulation of Aδ fibers no effect of CAPS on eEPSC (nor eIPSCs) was reported. In case of C fibers, lower concentrations of CAPS (0.2 μM) didn't influence the generation of eEPSCs, and the effect of more concentrated CAPS (1 μM) was blocked by simultaneous application of TRPV1 antagonist capsazepine. All together, these data show that TRPV1 agonists selectively inhibit eEPSCs induced by the activity of nociceptive C fibers in the spinal cord (Yang et al. 1999). Similar results were obtained from superficial DH neurons in young rats (P7 – P10), that consistently displayed an increase in sEPSC/mEPSC frequency and at the same time a significant reduction of the amplitude of eEPSCs as a result of CAPS treatment. The formation of eEPSCs was not impaired in neurons from deeper laminae III/IV, which corresponds with the absence of C fiber terminals. Interestingly, in newborn rats (P0 – P5) it was showed that although the acute application of CAPS (2 μM) abolished propagation of the signal in C fibers, it also caused such a strong increase in spontaneous glutamate release that the synaptic activity was sufficient to induce action potentials in postsynaptic neurons (Baccei et al. 2003). The CAPS inhibition of eEPSC can be explained by dose-dependent activation of TRPV1 in presynaptic terminals. While low concentration of TRPV1 agonists leads to a TRPV1-mediated increase in the Ca^{2+} level and therefore to stronger synaptic transmission, high concentration of agonists keeps the channels open, causing lasting depolarization that prevents any further synchronized release of neurotransmitters (Baccei et al. 2003).

TRPV1 agonists were also showed to modulate the frequency of inhibitory postsynaptic currents (IPSCs) in GABAergic and glycinergic interneurons of lamina II. In their early study, Wei and Zhao used immunohistochemical staining of the DH following an irritation of *n. tibialis* with CAPS and they suggested that the synaptic transmission between PAFs and inhibitory interneurons might be mediated by SP released from the presynaptic terminals upon TRPV1 activation (Wei and Zhao 1996). Later, the effect of TRPV1 agonists was confirmed directly at the first synapse by patch-clamp recordings from lamina II neurons. An acute application of CAPS (2 μM) led to an increase of both frequency and amplitude of spontaneous IPSCs (sIPSC) in 76% of measured cells, which were either purely GABAergic (72% of measured cells) or GABA/glycinergic (28% of measured cells). The effect of CAPS was blocked both by a selective TRPV1 antagonist SB366791 (10 μM) and by an antagonist of postsynaptic NK_1 receptors L732,128 (4 μM) (Ferrini et al. 2007).

In organotypically cultured mouse substantia gelatinosa neurons, the CAPS-induced increase of sIPSC frequency was also significantly reduced after the application of TTX, which indicates the importance of the activation of local spinal cord circuitry for the effect of CAPS (Ferrini et al. 2010).

4.3.2. The role of spinal TRPV1 during pathological pain states

Pathological states manifesting as chronic pain, such as peripheral neuropathy or chronic inflammation, represent an important health care issue and therefore receive continuous attention from scientific groups worldwide. Until now, the research of TRPV1 role in pathological pain states focused mainly on receptors expressed in peripheral nociceptive endings, as their function there is well documented and the site is easily accessible. Today, although it is assumed that many properties of peripheral TRPV1 can be also extended to central TRPV1, it is understood that TRPV1 in spinal cord plays a distinctive role in pathological nociception.

Following a peripheral tissue damage or inflammation, TRPV1 receptors were showed to undergo a significant sensitization, when the thermal activation threshold lowers to physiological values, so the receptors can be activated already by innocuous temperatures and even by the normal body temperature. Such sensitization leads to increased TRPV1 activity and therefore to heightened excitability of nociceptive PAFs, which represents one of the mechanisms of development of thermal hypersensitivity and allodynia. Sensitization of peripheral TRPV1 can be induced by many inflammatory mediators produced in affected tissues, such as bradykinin (Cesare and McNaughton 1996; Sugiura et al. 2002), prostaglandins (Moriyama et al. 2005), TNFα (Khan et al. 2008; Nicol et al. 1997), NGF (Bonnington and McNaughton 2003), protons (Caterina et al. 1997), or ATP (Tominaga et al. 2001). Some inflammatory substances are also suspected to modulate TRPV1 activity at the spinal cord level, and these will be discussed in more detail in Chapter 4.3.3.

The sensitization of TRPV1 is generally attributed to the degree of phosphorylation of intracellular parts of the receptor. It is well documented that TRPV1 receptors in DRG neurons are highly phosphorylated during peripheral inflammation and neuropathies (Cheng and Ji 2008), which is largely due to high concentrations of inflammatory mediators in the environment. Binding of inflammatory ligands triggers intracellular cascades of signalling molecules like protein kinase A and C (PKA, PKC), phospholipase C (PLC), Ca^{2+}-calmodulin dependent kinase II (CAMKII), mitogen activated protein kinase (MAPK), cyclin-dependent kinase 5 (Cdk5), or phosphoinositide 3 kinase (PI3K) (Suh and Oh 2005). The enzymes then phosphorylate specific serine and threonine residues in the intracellular part of TRPV1, causing its sensitization (Levine and Alessandri-Haber

2007). A more recent study using a spinal cord CGRP release assay confirmed that phosphorylation of TRPV1 influences the effectivity of TRPV1 agonist and antagonists (Mogg et al. 2013).

In general, TRPV1 is considered as the key mediator of the development of thermal hyperalgesia and allodynia that often accompany chronic pain states. Using a strain of *trpv1* knock-out mice, it was documented that the loss of TRPV1 receptor prevents the development of thermal hyperalgesia following chemically induced peripheral inflammation. At the same time, mechanical nociceptive sensitivity of these mice remained unchanged, as well as their response to acute noxious heat stimuli (Caterina et al. 2000; Davis et al. 2000), which might be explained by the presence of other high threshold heat receptors in C fiber afferents, such as TRPV2 (Caterina et al. 1999).

It is assumed that apart from the receptor sensitization to agonists, thermal hyperalgesia and allodynia can be also caused by TRPV1 upregulation. It was reported that during peripheral inflammation, an increased amount of TRPV1 can be found in DRG neurons, both in peripheral and central terminals. While in the periphery the increased number of TRPV1 resulted in stronger response to a stimulus, in the spinal cord it led to more robust release of neurotransmitters and neuromodulators (Luo et al. 2004; Tohda et al. 2001). During neuropathic states, a downregulation of TRPV1 occurs across the overall DRG population that can be at least partially explained by the degeneration of TRPV1+ PAFs (Lauria et al. 2006; Michael and Priestley 1999). However, a substantial increase of TRPV1 was reported in the DH in the CCI model of peripheral neuropathy (Kanai et al. 2005) and in the model of spinal cord injury (Wu et al. 2013), which suggests the involvement of spinal TRPV1 in the development of nociceptive disorders during these states. This assumption is supported by the finding that intrathecal application of TRPV1 antagonists leads to a substantial decrease of nociceptive hypersensitivity during peripheral inflammation or neuropathy. Kanai et al. reported that an intrathecal injection of TRPV1 antagonist BCTC alleviates mechanical allodynia in rats and its acute application on spinal cord slices prevents the CAPS-evoked release of SP and CGRP (Kanai et al. 2005). Also, intrathecal injections of TRPV1 antagonists was showed to effectively moderate thermal hyperalgesia during peripheral inflammation (Cui et al. 2006).

Surprisingly, following nerve damage, the upregulation of TRPV1 in the spinal cord was observed not only in affected fibers but also in surrounding uninjured neurons and even in neurons at different spinal levels (Hudson et al. 2001). Several explanations were suggested including the sensitization of neighbouring neurons by inflammatory mediators from recruited immune cells, or the signal propagation via collaterals and local spinal circuits (Choi et al. 2016). Such TRPV1 upregulation in unaffected neurons corresponds to the occurrence of secondary hyperalgesia that arises from uninjured neighbouring receptive fields under neuropathic conditions (Patel et al. 2011).

Pathological pain states are accompanied by specific changes at the spinal cord level, such as activation of glial cells and infiltration of immune cells, and many of those non-neuronal cells may also carry on their surface TRPV1 receptors (Marchand et al. 2005; Schomberg and Olson 2012). Using *trpv1* knock out mice, it was showed that TRPV1 probably plays an important role in microglia and astrocytes activation during peripheral neuropathy and inflammation, as these cells exhibited significantly lower degree of transformation in knock-out animals than in controls (Chen et al. 2009). The activated glial cells in their turn produce a broad variety of inflammatory mediators that further affect the nociceptive transmission in the spinal dorsal horn (Old and Malcangio 2012).

4.3.3. Modulators of the activity of spinal TRPV1

Unlike in the periphery, the receptors expressed in the spinal cord are hardly activated by high temperatures or low pH. However, their activity can be modulated by various endogenous substances that either interact directly with TRPV1 or act through cascades of second messengers after binding to their own receptors. Many inflammatory mediators can be found amongst these indirect TRPV1 modulators, and their enhanced production under pathological conditions usually leads to TRPV1 sensitization via phosphorylation and/or augmented incorporation to the cellular membrane (Malek et al. 2015).

One of the most versatile TRPV1 modulators is **adenosine triphosphate (ATP)**, an omnipresent molecule that can be found both in extra- and intra-cellular environment and is capable of direct binding to the receptor, as well as of its indirect modulation (Lishko et al. 2007; Tominaga et al. 2001). In cultured HEK293 cells expressing TRPV1 and in rat DRG neurons, it was reported that ATP interacts with metabotropic receptor $P2Y_1$ that activates intracellular PKC signalling cascade and through phosphorylation causes TRPV1 sensitization. In this study, the activation threshold of TRPV1 was reduced from 42 °C to 35 °C, which corresponds with development of thermal allodynia (Tominaga et al. 2001). Furthermore, Lishko et al. described direct ATP binding on TRPV1 N-terminus. When bound, ATP stabilizes the receptor and induces its phosphorylation, which together leads to its sensitization (Lishko et al. 2007; Phelps et al. 2010).

The ATP binding site can also competitively interact with **calmodulin (CaM)**, a highly conserved protein comprising of two flexibly connected lobes, which each can bind two Ca^{2+} ions (Vetter and Leclerc 2003). Since binding of Ca^{2+}/CaM complex leads to the desensitization of TRPV1, the dynamic balance between ATP and Ca^{2+}/CaM binding often underlies the immediate activity of the receptor (Lau et al. 2012; Lishko et al. 2007). At the same time, another CaM binding site was localized on TRPV1 C-terminus using *in vitro* binding assays (Numazaki et al. 2003),

whose deletion or mutation was also showed to substantially reduce TRPV1 desensitization following CAPS stimulation (Grycova et al. 2008). This binding site has higher affinity for CaM and is likely involved in separate regulatory mechanisms (Lau et al. 2012).

In the present, a great deal of attention concentrates on **phosphatidylinositol 4,5-bisphosphate (PIP_2)**, a membrane lipid that also participates on both direct and indirect modulation of TRPV1 activity. So far, several PIP2 binding sites were discovered at TRPV1, one on the N-terminus and two on the C-terminus, that all partially overlap with CaM binding sites (Grycova et al. 2012). With the help of *in vitro* binding assays, it was demonstrated that the proximal C-terminal TRPV1 region is sufficient for PIP_2 binding (Ufret-Vincenty et al. 2011), although PIP_2 interaction with the distal region was showed to interfere with CaM binding to TRPV1 (Grycova et al. 2012). Originally, PIP_2 was believed to act as a tonic inhibitor that prevents TRPV1 activation when bound and that can be cleaved away by PLC in order to allow TRPV1 activation (Chuang et al. 2001). However, other studies have showed that depletion of PIP_2 leads to TRPV1 deactivation (Yao and Qin 2009) and that application of PIP_2 to inside-out membrane patches enhances TRPV1 activity (Lukacs et al. 2007), all together indicating that PIP_2 acts as an activator rather than an inhibitor. Finally, using high resolution crystallography of TRPV1 located in lipid nanodiscs, Gao et al. described PIP_2 as a tightly bound co-factor that stabilizes TRPV1 in its resting state, which can both inhibit and potentiate further receptor activation depending on current conditions (Gao et al. 2016).

Indirect TRPV1 modulators represent a diverse, numerous group of endogenous substances that can be upregulated during pathological states and contribute to TRPV1 sensitization via specific signalling cascades. Here, we focus on the best established inflammatory mediators, although it is necessary to mention that TRPV1 can be indirectly modulated also by substances without primary inflammatory function including protons (Davis et al. 2000) or glutamate (Kim et al. 2009).

One of the best known TRPV1 modulators is **bradykinin (BK)**, a nonapeptide inflammatory mediator produced by damaged neurons that acts through its receptors B1 and B2 (Scholz and Woolf 2007). When Malek et al. analyzed levels of B1 and B2 mRNA in DRG neurons from rats with CCI, they observed upregulated transcription of B2, but not B1 (Malek et al. 2015). Since activation of the B2 receptor promotes via PLA_2 pathway production of lipoxygenase metabolites (AEA, OEA, PEA) that act as endogenous TRPV1 ligands (Petcu et al. 2008; Starowicz et al. 2013), it was suggested that this might be one of the mechanisms by which BK induces thermal hypersensitivity and allodynia. The activation of B2 receptor also triggers PKCε signalling pathway, which leads to TRPV1 phosphorylation and sensitization (Spicarova and Palecek 2009). Using TRPV1-expressing HEK293 cells and rat DRG neurons, BK was showed to lower the TRPV1 activation threshold to physiological values in a PKCε-dependent way (Cesare and

McNaughton 1996; Sugiura et al. 2002). Furthermore, peripheral application of inhibitors of PLC, PKC, PLA_2 and lipoxygenases resulted in reduction of pro-nociceptive effects of BK (Ferreira et al. 2004).

Many inflammatory chemokines are also implicated in indirect modulation of TRPV1, such as **chemokine CCL2** that will be in more detail described in Chapter 6. From the cytokine family, special attention was lately payed to **cytokine TNFα**, since the first *in vitro* experiments in cultured DRG neurons showed that application of TNFα potentiates the neuronal response to CAPS and suggested that TNFα acts through its TNFR receptors (Constantin et al. 2008). Soon after, the modulatory effect of TNFα was documented also in neurons from spinal cord slices in naïve rats (Spicarova and Palecek 2010) and in a model of peripheral neuropathy (Spicarova et al. 2011). In both cases, the superficial DH neurons reacted to application of TNFα by an increase in sEPSC and mEPSC frequency and at the same time showed higher sensitivity to endogenous TRPV1 agonists. Park et al. observed the same effect of TNFα application in spinal neurons that were identified as excitatory neurons carrying glutamate transporter vGluT-2. The group also recorded EPSC frequencies in neurons from *trpv1* knock-out mice that showed no response to TNFα, suggesting that its effect is indeed mediated by TRPV1 (Park et al. 2011a). When TNFα mRNA level was measured in the CCI model, an upregulation was documented in DRG neurons in the beginning of the neuropathy development but not in later stages, indicating that TNFα might contribute to the hypersensitivity during the transition from acute to neuropathic pain (Malek et al. 2015).

Prostaglandins (PG) form another big group of potential TRPV1 modulators that are derived from arachidonic acid and influence TRPV1 activity via their own metabotropic receptors associated with G proteins. The most implicated in the development and maintenance of chronic pain are prostaglandins PGE_2 and PGI_2 that act through several receptors. Activation of receptor EP1 stimulates PLC that in its turn mobilizes Ca^{2+} reserves and induces TRPV1 phosphorylation via PKC. Receptors EP2 - 4 modulate the function of enzyme adenylate cyclase (AC), regulate generation of cAMP and thus activation of PKA. Similarly, activation of the PI receptor triggers the AC – cAMP – PKA cascade (Kawabata 2011; Narumiya et al. 1999). The kinases-mediated sensitization results in lowering of the TRPV1 activation threshold to 35 °C (Moriyama et al. 2005).

4.3.4. Clinical use of ligands and modulators of spinal TRPV1

For patients suffering from chronic pain, opioid analgesics are still considered as the most effective treatment, although their side effects are well-known and often account for the failure of the therapy. Therefore, a safer alternative for heavy opioid users is urgently needed. TRPV1 ligands

and modulators represent a group of potential clinical targets for chronic pain management, because they directly influence TRPV1 activity in the spinal cord and thus the strength of nociceptive synaptic transmission. According to their specific effect on TRPV1, these substances offer several possible ways of medical use.

Based on their successful employment in the periphery, it was suggested that powerful TRPV1 agonists, such as CAPS or RTX, might be administered intrathecally in order to functionally deactivate central branches, while preserving the bodies and peripheral terminals of nociceptive DRG neurons. When tested in animals, an intrathecal injection of RTX had a very mild (Mishra and Hoon 2010) or no effect on acute thermal perception (Jeffry et al. 2009) due to the intact peripheral sensitivity. However, when administered to animals with peripheral inflammation, RTX application led to a dramatic reduction of thermal hypersensitivity while the mechanical sensitivity was maintained (Jeffry et al. 2009; Mishra and Hoon 2010). In a similar way, the use of TRPV1 antagonists might be promising when applied locally in in the spinal channel, whereas systemic administration is problematic (Xia et al. 2011). Also, it was showed that it is possible to use positive allosteric modulators like MRS1477 to sensitize spinal TRPV1 receptors to such extent that their subsequent hyperactivity leads to a calcium overload in presynaptic buttons. High intracellular Ca^{2+} levels then prevent any further synaptic transmission, which results in selective analgesia (Kaszas et al. 2012; Lebovitz et al. 2012).

Another possible approach is to downregulate TRPV1 in central terminals of PAFs. This can be achieved by blocking incorporation of spinal TRPV1 into the presynaptic plasmatic membrane by interfering with some of the many enzymes and mediators involved in the process. For example, Liu et al. documented, that phosphorylation of TRPV1 threonine 407 by cyclin-dependent kinase 5 (Cdk5) plays an important role in the transport of the receptor to the membrane. Furthermore, the group reported that intrathecal administration of a design peptide that prevents the phosphorylation reduces the surface level of presynaptic TRPV1 and successfully moderates heat hyperalgesia and inflammatory pain in rats (Liu et al. 2015). The overall level of TRPV1 can be also lowered by inhibiting directly the TRPV1 expression in DRG neurons, for example with the help of RNAi. Following an intrathecal injection of anti-TRPV1 siRNA, more than 50% decrease in cold allodynia was observed in a rat model of peripheral neuropathy over 5 days. Furthermore, siRNA administration had a significant analgesic effect on spontaneous visceral pain behaviour in reaction to a CAPS application to the rectum in mice (Christoph et al. 2006).

Other groups explored the interaction of TRPV1 and a cytoskeletal A kinase anchoring protein 79 (AKAP79) that binds both TRPV1 and enzymes PKC, PKA and calcineurin, and forms with them a functional signalling complex (Zhang et al. 2008). Using immunohistochemistry,

calcium imaging and patch clamp recordings from secondary sensory neurons in mice, Fisher et al. confirmed that selective blocking of AKAP79/TRPV1/enzyme complex by a short antagonist peptide is effective in alleviation of chronic pain in animal models (Fischer et al. 2013).

5. THE ROLE OF μ-OPIOID RECEPTOR IN NOCICEPTION AND PAIN TREATMENT

5.1. Opioid receptors

Opioid receptors belong to a superfamily of G protein-coupled receptors (GPCRs) and further to a family of rhodopsin receptors (A family). Their molecules contain 7 transmembrane α-helixes that form a helix-like structure with a central cavity partially covered by variable extracellular loops and an extracellular N-terminus with multiple glycosylation sites. All members of this family share about 60% sequence homology, the transmembrane sections being the most conserved parts. The extracellular structures show the biggest variability and thus play the key role in ligand selectivity of the receptor (Law et al. 2000).

According to their unique pharmacological responses to specific ligands, opioid receptors can be further divided into four subtypes. So-called 'classical' opioid receptors include μ- (or MOP-R, where M stands for morphine), the κ- (or KOP-R, where K stands for ketocyclazocine) and the δ-opioid receptors (or DOP-R, where D is based on vas deferens, a site in mouse where the receptor was first identified). More recently a fourth 'non-classical' subtype was discovered and named based on its preferential endogenous ligand the nociceptin/orphanin FQ receptor (or NOP-R) (Waldhoer et al. 2004; Zöllner and Stein 2007).

In mammals, opioid receptors have been found in cells of the central and peripheral nervous system, in the immune system and in the endocrine system. Particularly high density of opioid receptors have been observed in those parts of nervous system that are involved in processing and integration of pain sensation – the brainstem, the medial thalamus, the hypothalamus, the limbic system and the spinal cord. The expression of each type of opioid receptors depends on the type of neural pathway. In ascending pathways of the spinal cord, μ-, δ- and κ-opioid receptors are all expressed in DRG neurons, the spinal cord gray matter and in the spinal trigeminal ganglion, whereas predominantly μ- and κ-receptors are detected in thalamus. In parallel with that, μ- and κ-receptors are mostly found in higher levels of descending pathways, such as in the periaqueductal gray and raphe nuclei, while the lower levels contain all μ-, δ- and κ-receptors (Mansour et al. 1995). The distribution of nociceptin/orphanin FQ receptor seems to be widespread in central nervous system, both in major brain areas and in neurons of ventral and dorsal horn of the spinal cord, suggesting its involvement in many neural functions, including nociception (Neal et al. 1999).

Opioid receptors respond to both endogenous and exogenous ligands. Endogenous opioid receptors ligands form four major groups: endorphins, enkephalins, dynorphins and endomorphins, all molecules with a peptide structure that almost exclusively behave like agonists. Endogenous

opioids share a specific sequence of amino acids (Tyr(Phe)-Gly-Gly-Phe-Met/Leu) called the opioid motif, which is followed by a variable C-terminal chain. They are mostly derived from four possible precursors (pro-opiomelanocortin, proenkephalin, prodynorphin, and pronociceptin / orphanin FQ) that subsequently undergo complex cleavage and post-translational modifications (Koneru et al. 2009). Exogenous ligands of opioid receptors are described by their relation to morphine, naturally occurring in opium. They count either among semi-synthetic substances with modified morphine structure (such as diacetylmorphine = heroin) or among fully synthetic derivatives that often show no morphine resemblance. Amongst these substances, both agonists (such as naloxone and naltrexone) and antagonists (such as oxycodone and fentanyl) of opioid receptors can be found, some of them with general effect and some of them showing strong receptor specificity (Koneru et al. 2009; McDonald and Lambert 2011; Wikipedia 2016)

Both endogenous and exogenous opioid ligands act through an opioid receptor on the same intracellular effector cascade and the process is triggered by dissociation of a pertussis toxin-sensitive G protein (G_i/G_o) coupled to the receptor. These G proteins are formed by three distinct subunits (α, β and γ) that have many sequence variations and therefore allow a wide range of possible G proteins to be engaged. An inactive α-unit binds a molecule of guanosine diphosphate (GDP) which is then dissociated in response to receptor activation and a molecule of guanosine triphosphate (GTP) binds in its place. Subsequently, the Gα-GTP unit splits from the Gβγ complex and both are able to influence different intracellular pathways (McDonald and Lambert 2011).

The best explored effect of opioid receptors activation is the inhibition of voltage-gated calcium channels directly through the Gβγ complex. It targets the N- and P/Q-type Ca^{2+} channels that are located in the membrane of neuronal synaptic terminals and participate there in the transmitter release during synaptic transmission. The binding of the Gβγ to various Ca^{2+} channel subunits causes increased voltage dependence of the channel and its slower activation which leads to lower frequency of the pore opening (Al-Hasani and Bruchas 2011; Bourinet et al. 1996). The released Gβγ complex is also known to bind to G protein-gated inward rectifying potassium channels (GIRKs) and to facilitate their activation. These potassium channels are tetramers composed of members of the GIRK protein family that are expressed in neurons throughout the CNS. Their increased activity augments the K^+ intake, leading to the membrane hyperpolarization and therefore to reduced neuronal excitability (Sadja et al. 2003; Torrecilla et al. 2002).

Activation of opioid receptors has been also showed to lead to inhibition of adenylyl cyclase (AC) by its interaction with the dissociated Gα subunit, which results in decreased production of cyclic adenosine monophosphate (cAMP). cAMP is a versatile mediator that is able to enhance the hyperpolarization-activated K^+ current (I_h) in a neuronal cell, causing its faster repolarization and

therefore increased excitability. Opioid-induced lowering of intracellular cAMP levels thus contributes to a slower firing rate of the target neuron (McDonald and Lambert 2011).

The inhibition of voltage-gated Ca^{2+} channels and the AC together with the activation of GIRK channels result in decreased neuronal excitability, diminished transmitter release at synaptic terminals and a slower firing rate. Thus the activation of opioid receptors located in neurons of the nociceptive pathway directly inhibits nociceptive signalling, hence the analgesic effect (Waldhoer et al. 2004). At the spinal cord level, this effect is likely mediated both by presynaptic reduction of primary afferent input and by postsynaptic inhibition of DH neurons (Kohno et al. 1999).

5.2. μ-opioid receptor in nociception

5.2.1. Structure and distribution of μ-opioid receptor

The μ-opioid receptor (MOP-R) was identified in 1993 (Chen et al. 1993; Eppler et al. 1993) and so far it's been successfully cloned from rat, mouse, human, pig and other vertebrae (Waldhoer et al. 2004). Even before the receptor structure was known, more than one subtype of MOP-R were suspected based on clinical experience with different responses to various opioid drugs. Soon a second opioid binding site was discovered and selective blocking of both sites then showed diverse localization of MOP-R subtypes and their distinctive selectivity profile (Ling and Pasternak 1983; Lutz et al. 1985; Paul et al. 1989). Later on, the MOP-R gene was localized in various animals including human where it's found on the short arm of the 6th chromosome (HGNC 2016). It comprises of four exons from which three are encoding the transmembrane section along with the N-terminal domain and are analogous to the exons of DOP-R and KOP-R. The fourth exon that is unique for MOP-R encodes an intracellular 12 AA chain of the C-terminus. Since its first detection, a number of splice variants of MOP-R gene has been isolated from mice, rats and humans, all of them functional and showing distinct spatial features (Pasternak 2014). For example, the splice variant MOP-R1 can be found both in presynaptic and postsynaptic terminals, whilst the variant MOP-R1C is exclusively located presynaptically (Abbadie et al. 2000). Individual splice variants usually show greatest variability in the intracellular C-terminus, the site of signal transduction, and therefore they bind the same ligands with different effectivity. The functional assessment of some of the splice variants in GTPγS stimulation assays showed that the maximal stimulation varied significantly among the drugs and the splice variants, for example b-endorphin can act as a potent agonist of higher efficacy than DAMGO when it interacts with the MOP-R1E variant, yet it acts as

a partial agonist (with only 44% of the efficacy of DAMGO) on the MOP-R1C variant (Bolan et al. 2004; Pasternak 2004).

Recently the molecular structure of mouse MOP-R was described by using membrane crystallography. In their study, Manglik at al. analyzed the crystals of MOP-R bound to the irreversible antagonist β-funaltrexamine and revealed an unusually exposed ligand-binding pocket that is easily approached and therefore allows rapid turnover even in case of a very potent ligand (Manglik et al. 2012; see Figure 5.1). Furthermore, crystallography is appreciated for casting light on ligand–receptor interactions both at the orthosteric binding site (such as the binding site of endogenous opioid ligands) and allosteric binding sites (such as the sodium binding site newly described in DOP-R and strongly suspected in other receptors (Fenalti et al. 2014)). The precise model of MOP-R structure is a promising step in discovering new effective drugs, however it is necessary to realize that MOP-Rs are dynamic systems and that their temporary conformation and currently bound ligands can significantly influence its behaviour (Shang and Filizola 2015).

The tertiary structure of MOP-Rs is still slightly mysterious. In crystallography studies, they were observed to crystalize in dimers, forming a two-fold symmetrical structure connected by trans-membrane helixes 5 and 6. However, such an arrangement would probably prevent successful binding of a G protein to the receptor and might be therefore considered a mere artefact (Manglik et al. 2012). It was indeed proven that the oligomerization of MOP-Rs is not required for ligand binding and that the monomeric receptor represents a functional unit capable to trigger the G protein dissociation (Kuszak et al. 2009). On the other hand, there is a lot of data supporting the existence of functional heterodimers of different opioid receptors, from which the most interesting would be dimers of MOP-R and DOP-R. Most of the evidence is based on pharmacological profiles of the cells expressing both types of opioid receptors and it shows that supposed heterodimerization results in altered ligand selectivity and efficacy (Jordan and Devi 1999; Law et al. 2013).

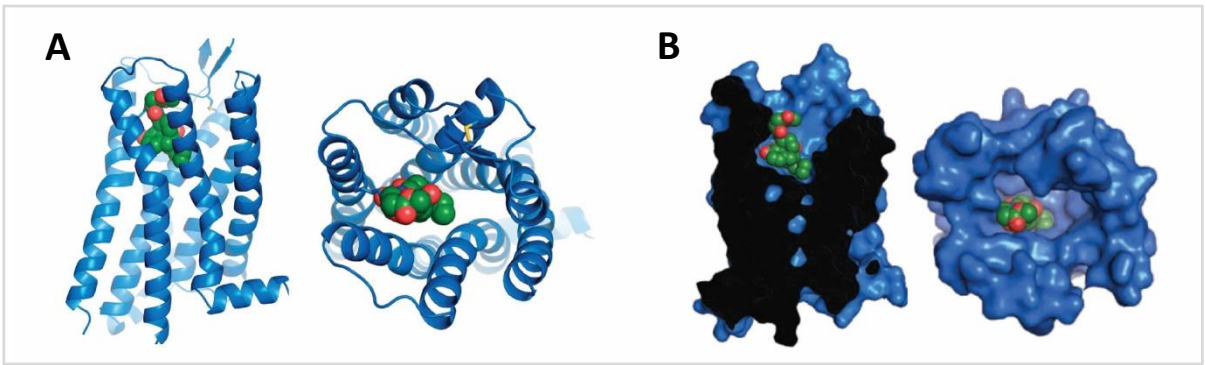

Figure 5.1: Crystal structure of MOP-R determined with β-funaltrexamine covalently attached in the binding pocket. (**A**) Side and top view, ribbon model (**B**) Side and top view, molecular model (adapted from Manglik et al. 2012).

Within a neuronal cell, MOP-Rs can be found incorporated in the plasmatic membrane where they sometimes tend to gather in specific restricted areas. With the help of FRAP analysis, this departmentalization was showed to be dependent on the type of ligand bound to the receptor - for example, morphine-bound MOP-Rs were showed to rather cluster in membrane rafts, whereas DAMGO-bound MOP-Rs were in some cases freely dispersed (Sauliere-Nzeh et al. 2010). In general, MOP-Rs were observed both in somato-dendritic (postsynaptic) and axonal (presynaptic) neuronal regions where they play slightly different roles. The activation of somatic MOP-Rs results in decreased neuronal excitability whereas presynaptic MOP-Rs response leads to the inhibition of neurotransmitter release (Williams et al. 2001).

When it comes to the whole nociceptive neuronal network, MOP-Rs were detected in all relevant parts of human CNS. At the supraspinal level, MOP-Rs were at first visualized *in situ* by hybridization immunohistochemistry and found mostly in the cortex (especially in the layer V) and to lesser extent in other brain areas (Peckys and Landwehrmeyer 1999). Later it became evident that different MOP-R splice variants can have distinct location in the brain, as was demonstrated in rats. It was determined that the isoforms MOP-R1, MOP-R1A and MOP-R1B are present in all relevant brain areas with light regional preference, whereas the isoforms MOP-R1C, MOP-R1C1, MOP-R1C2 and MOP-R1D are almost undetectable (Oldfield et al. 2008).

In the spinal cord, MOP-Rs have been detected in the DH, both in postsynaptic membrane of DH neurons and in presynaptic terminals of PAFs coming from sensory ganglions (DRG, TG), with the strongest hybridization signal detected in DH lamina II (Peckys and Landwehrmeyer 1999). Postsynaptic MOP-Rs are there mostly expressed by non-GABAergic neurons that comprise approximately 10% of the local neuronal population and might represent a population of excitatory inter-neurons. These MOP-R-positive cells were observed to form connections with two populations of afferents – the ones containing substance P and also the unmyelinated non-peptidergic C fiber terminals, both of them being known to play a part in nociceptive transmission. Opiate binding to MOP-Rs of the DH neurons would therefore lead to an inhibition of the nociceptive pathway (Spike et al. 2002). In rat, the presence of MOP-R was also documented in dendrites of lamina I projecting neurons in the trigeminal dorsal horn that participate in nociceptive transmission towards the thalamus and parabrachial nuclei (Mitchell et al. 2004).

In rat DRG, MOP-Rs were found in small-, medium-, and large-diameter neurons (Mansour et al. 1994). Approximately half of all nociceptive neurons, predominantly small and medium cells, were MOP-R-positive and responded to opioids (Rau et al. 2005; Silbert et al. 2003). In these neurons, MOP-R was co-expressed with classical sensory neuropeptides such as SP and CGRP (Li et al. 1998). The number of MOP-Rs present in DRG neuronal populations changes during the

ontogenetic development, as was showed in mice. Immediately after the birth, MOP-Rs are present in DRG neurons of all sizes, whereas on the 27th postnatal day they are downregulated in large-diameter neurons and prevail in small- and medium-sized neurons (Beland and Fitzgerald 2001).

5.2.2. Agonists of μ-opioid receptor in pain management

As was established earlier, MOP-R can be found in neurons of the nociceptive pathway and through its activation opioids are able to influence perception of pain. MOP-R agonists are still considered to be the most effective analgesics in treatment of severe acute, post-surgical, and cancer pain. In spite of their various side-effects (which are described later in this chapter), the worldwide usage of opioids continues to grow – for example in Czech Republic the morphine consumption increased from 51 kg in 2009 to 57 kg in 2013 and for oxycodone the numbers are even higher (INCB 2015). And together with these numbers rises also the need for new, safer analgesic drugs.

Morphine and **codeine** are two opioids with alkaloid structure that occur naturally in the poppy plant and throughout the human history they were extracted, used in medicine and enjoyed for their psychotropic effects. Even though their source is mostly exogenous, trace amounts of morphine were also detected in various mammal tissues (Grobe et al. 2010). Originally, their presence was explained as a trace of the intake with food, however now there is evidence that morphine and his derivatives can be synthetized *de novo* in mammal cell cultures (Poeaknapo et al. 2004), as well as *in vivo* (Grobe et al. 2010). The morphine molecule ($C_{17}H_{19}NO_3$) has a complicated five-ring structure with an aromatic core. Its 3-hydroxyl group is the essential site for morphine affinity to MOP-R (Pert et al. 1973), whereas the 6th position can be variously modified, often creating even more potent morphine derivatives, such as morphine-6β-glucuronide (Pasternak et al. 1987). The analgesic effects of morphine were long known and even before its structure was properly described, its first analogs were synthetized – one of them being heroin (diacetylmorphine). Using an *Oprm1* knock-out mice, it was proven that the morphine-induced effects including analgesia are mediated by the MOP-R (Matthes et al. 1996). To lesser extent, morphine can also bind to KOP-R and DOP-R with supportive analgesic effect and some studies even suggest its binding to MOP-R/DOP-R heteromers (He and Lee 1998; Yekkirala et al. 2012).

It was established that substances that are able to activate MOP-R and cause an effect similar to morphine are called opioids, regardless of their site of production that could be either endogenous or exogenous. **Endogenous opioids** are expressed in certain populations of DRG sensory neurons that are often involved in the nociceptive pathway and that were showed to localize their opioids in both central and peripheral terminals (Stein et al. 2009). The MOP-R has several selective

endogenous ligands from which we will mention the most important ones. **B-endorphin** is a 31 AA long peptide that is cleaved from its precursor proopiomelanocortin (POMC). It was originally found in the pituitary gland (Li and Chung 1976) and in the *nucleus arcuatus* of the hypothalamus (Bugnon et al. 1979). From there, β-endorphin expressing neurons project into many other brain areas implicated in pain processing. In addition, β-endorphin was also detected in neurons of the *nucleus tractus solitarii* in the caudal medulla, whose connections were showed to enter the spinal cord (Bronstein et al. 1992). Another endogenous opioid, **met-enkephalin**, is a pentapeptide that occurs naturally as a splice product of proenkephalin and its expression was documented in neurons of various brain areas, as well as in the periaqueductal gray and the DH of the spinal cord (Akil et al. 1984). Met-enkephalin has been showed to activate both DOP-R and MOP-R and apart from its neuromodulatory effects, it has been closely associated with cell regeneration and development (Akil et al. 1984; Zagon et al. 1999). More recently, two additional endogenous opioids with higher MOP-R affinity and selectivity were found – tetrapeptides **endomorphin-1 and -2**. While endomorphin-1 is predominantly distributed throughout the brain and the upper brainstem, endomorphin-2 is mostly found in the spinal cord and the lower brainstem, and both of them were found to play a role in pain transmission (Koneru et al. 2009).

On the other side of the spectrum stand **exogenous opioids.** In many cases they are products of artificial chemical modification of the basic morphine structure, when most effective were alternations of the 6th/14th position (generating potent analgesics like hydromorphone, hydrocodone, metopon, and oxycodone) and reduction of the C7–C8 double bond (resulting in dihydromorphine). The 6th position changes also generated a number of MOP-R antagonists (such as β-funaltrexamine, naloxonazine, and naloxazone) that are now widely used in laboratories in the research of opioid mechanisms and serve further as basis for development of new agents (Pasternak and Pan 2013). Other opiates can be divided into several classes with chemical structure more or less distinct from morphine. Morphinans, represented by two agonists levorphanol and butorphanol and one antagonist levallorphan, are based on modified morphine molecule without double-bonds and without the 6-hydroxyl group, which leads to the loss of receptor selectivity. Oripavines include active oripavine derivatives (such as etorphine, diprenorphine, and buprenorphine) that are non-selective and are often clinically used. Lastly, there is a large group of opioid alkaloids that are completely synthetic and their chemical structure can be very variable. This group include some of the best known drugs, such as meperidine (a 4-phenylpiperidine) and MOP-R highly selective methadone and meptazinol (Pasternak and Pan 2013).

Apart from opioid alkaloids, synthetic MOP-R agonists can be as well based on the structure of natural endogenous opioid peptides. To prevent those substances from rapid deterioration, it was

necessary to substitute a D-amino acid in the 2nd position (usually D-Ala) (Pert et al. 1976). This improvement led to the development of a number of opioids with variable receptor selectivity and efficacy, from which we will mention a potent MOP-R agonist, **[D-Ala^2,N-MePhe^4,Gly-ol^5] enkephalin** (DAMGO). DAMGO is highly selective MOP-R agonist that is widely used in research (for MOP-R labeling or in electrophysiology, like in this work) and in clinical pharmacology (Handa et al. 1981; Seki et al. 1998).

The analgesic effect of MOP-R agonists is accompanied by multiple side effects. The most common one is the respiratory depression, a result of activation of MOP-Rs within the medullar respiratory centres that leads to decreased sensitivity of local chemoreceptors to carbon dioxide. Nausea and vomiting can also occur as a result of the stimulation of the medullar chemoreceptor zone. Furthermore, opioid drugs are known to act on nerve fibers of the myenteric plexus causing a visceral smooth muscle contraction that leads to constipation and worse absorption through the gut wall (McDonald and Lambert 2011). To lighten these side-effects, opioid receptor antagonists, such as methylnaltrexone, have been successfully used as they can be administered subcutaneously and act only peripherally (they don't pass the hemato-encephalic barrier) (Sinatra 2006).

Another serious side-effect of opioid use is the possibility of the development of **addiction**. Addiction is defined on the basis of behavioural abnormalities occurring during chronic opioid treatment, such as loss of control and compulsive drug intake despite damaging consequences (Nestler 2004). The drug becomes necessary to avoid the development of withdrawal syndromes that often develop in opioid users after the drug supply is interrupted (Law et al. 2013). According to the current opinion, addiction occurs as a result of alteration of neuronal circuits associated with stress, anxiety, memory, and reward, where it acts through positive reinforcement. It can be therefore considered as a result of drug-induced neural plasticity (Waldhoer et al. 2004).

Satisfactory analgesic effect is further limited by the development of MOP-R desensitization and overall **opioid tolerance**. Immediately after the binding of a ligand to MOP-R, mechanisms responsible for acute receptor desensitization come into the play, MOP-Rs are phosphorylated by G protein-coupled receptor kinase (GRK) and consequently bound by protein arrestin. In approx. 5 minutes, an equilibrium is achieved when the desensitization mechanisms are in balance with sensitization mechanisms, such as the receptor dephosphorylation at the cell surface. During following hours, the development of short-term tolerance can be observed as a result of endocytosis of surface receptors that is to some extent compensated by receptor recycling (Williams et al. 2013). The long-term opioid tolerance occurring as a consequence of chronic opioid treatment is a more complex process that likely depends on multiple regulatory mechanisms and takes place at the level of individual receptors, at the level of a single neuron and at the level of the whole

opioid-sensitive neural network. It is described as reduced general responsiveness to opioid agonists usually leading to increased doses in order to achieve the desired effect. The development of opioid tolerance can be mostly explained by the effort of opioid-responsive neurons to keep their homeostasis and to normalize their activity while employing both adaptive and counter-adaptive processes. The adaptations are regulatory processes that lead to reduced receptor response or sensitivity, while the counter-adaptations refer to processes that diminish opioid responses by triggering opposing regulatory mechanisms and intracellular pathways (Christie 2008; Williams et al. 2013).

However helpful in pain management, MOP-R agonists had been also reported to induce abnormal pain, leading to the development to so-called **opioid-induced hyperalgesia (OIH)** (Angst and Clark 2006). Early clinical observations were later supported by targeted studies in patients undergoing anesthesia or post-operative pain treatment (Fletcher and Martinez 2014). Subjectively, the patients distinguish the original underlying pain and the pain occurring as a consequence of the treatment itself, both in quality and in location (Devulder 1997). Interestingly, it appears that the heighten sensitivity is selective for a specific kind of noxious stimuli – for example, in a study in patients with chronic pain opioid treatment (methadone, morphine), a hyperalgesic reaction occurred during cold pressor testing but not during electrical stimulation (Hay et al. 2009). In pre-clinical research, numerous studies concentrated on animal models of OIH. The first opioid analgesia in rats/mice lasts for several hours and subsequently a state of lowered nociceptive threshold develops that prevails for several days (Minville et al. 2010; Celerier et al. 2000; Van Elstraete et al. 2005). Furthermore, the extent of sensitization was showed to be in direct correlation with a dosage of the drug (Cabanero et al. 2009).

The gradually altered pain perception with a lower pain threshold occurs at the same time as the development of analgesic tolerance, leading together to the need of an increased dosage of the drug. It was therefore suggested that these two phenomena might share some of the underlying molecular mechanisms (Christie 2008). Several cellular processes were so far implicated in OIH development including the modulation of spinal TRPV1 activity, which is further discussed in the following chapter.

5.3. TRPV1 and opioid-induced hyperalgesia

As it was said in the previous chapter, the development of OIH and opioid tolerance leads to progressive augmentation of the effective drug dose and can ultimately result in a complete failure of the pain treatment. Therefore, the mechanisms underlying OIH had been in the centre of attention of numerous scientific groups.

In the beginning, a widely researched topic was the presence of toxic opioid metabolites following the opioid treatment that can cause neural irritation and therefore both hypersensitivity and high-dose tolerance. Indeed, substances like morphine-3-glucoronide (Lewis et al. 2010) and hydromorphone 3-glucuronide (Smith 2000) were showed to have neurotoxic activity and in certain cases, the accumulation of these agents in cerebro-spinal fluid was quite significant (Mitra 2007). Later, it was suggested that glutaminergic system alternations might also play an important role and it was documented that repeated morphine treatment can trigger apoptotic processes via NMDA receptors in DH neurons (Mao et al. 2002). Furthermore, morphine administration was also showed to elicit an increased expression of other neurotransmitters, such as SP and CGRP, in DRG neurons. These substances are released upon nociceptive stimulation and their higher concentration results in stronger postsynaptic response and thus hypersensitivity (Mao et al. 1995; Ossipov et al. 2005).

More recently, several studies have linked the OIH development with activity of cation channel TRPV1 located in the spinal cord. As was established earlier, MOP-Rs are in the DH located both pre- and postsynaptically. A subset of MOP-R afferents also express TRPV1 receptors and these are assumed to participate in thermal nociception. After this neuronal population was destroyed by an ultra-potent TRPV1 agonist RTX in rats chronically treated with morphine, the subject animals did not develop opioid tolerance and in comparison with the control group they didn't show any long-term decrease in MOP-R – G protein coupling (Chen et al. 2007a). Another research group subsequently showed that in rats chronically treated with morphine the TRPV1 immunoreactivity increases in DRG neurons, neurons of DH, and in the sciatic nerve. When pre-treated with an intrathecal injection of a selective TRPV1 antagonist SB366791, neither morphine tolerance nor thermal hyperalgesia developed (Chen et al. 2008). These findings were also supported by an experiment comparing wild-type and *trpv1* knock-out mice that were chronically treated with morphine. While the subjects of the control group manifested thermal and tactile hyperalgesia, the *trpv1* knock-out mice failed to show any sign of hypersensitivity. Moreover, when TRPV1 antagonist AMG 0347 was administered orally to the subjects with already developed hyperalgesia, a reversal of hypersensitivity was observed (Vardanyan et al. 2009).

A study conveyed by Zhou et al. documented a substantial hyperactivity in the lamina II neurons following acute administration of MOP-R agonist DAMGO. Slices of rat spinal cord were used and the neuronal activity was assessed based on electrophysiological recordings of evoked and miniature EPSCs. In the target neurons, the initial inhibition of activity followed shortly after brief (3 minutes) application of DAMGO and then a gradual increase in activity was observed during the wash-out phase (25 minutes). In approximately half of the neurons this increase reached higher values than in the beginning of the experiment, showing the possible development of hyperalgesia (Zhou et al. 2010). Moreover, this study states that TRPV1-expressing primary afferents are critical for opioid-induced LTP in the spinal cord. The population of TRPV1-positive PAFs was selectively destroyed in target animals by the RTX treatment, which prevented the activity increase during the wash-out phase and to prolongation of DAMGO induced inhibition (Zhou et al. 2010).

The presented evidence therefore suggests that TRPV1 channels play a role in development and maintenance of opioid-induced hyperalgesia at the spinal cord level. The presynaptic terminals of TRPV1-positive DRG neurons represent the site of the greatest interest, however the exact mechanism of TRPV1 involvement and/or its possible interaction with MOP-R are still not clarified.

6. CHEMOKINE CCL2 AS A NEUROMODULATOR

6.1. Chemokines and chemokine receptors

Chemokines are peptides usually constituted by 70 – 100 AA residues with molecular weight of 8–14 kDa. Their name stands for **chemo**tactic cyto**kines**, describing their ability to selectively attract and activate monocytes, neutrophils and lymphocytes. The family of human chemokines represents the most numerous cytokine group, as it includes more than 50 molecules that show among themselves 20–95 % sequential homology (Gao and Ji 2010). Chemokines are secreted by several types of cells in response to various signals including other inflammatory cytokines, and once released, they form a chemical gradient (known as chemokine gradient) along which the cells carrying the relevant chemokine receptor migrate towards the site of highest chemokine concentration (Callewaere et al. 2007).

We recognize four chemokine sub-families (CC, CXC, XC and CX3C), in which particular chemokines are numbered CXCLn, CCLn, XCLn and CX3CLn. The sub-families are distinguished by the number and location of cysteine residues in the N-terminal region of the molecule, which form disulfide bonds in between and may be interspersed with other AAs. Except of XC family that has only two, all chemokines contain 4 cysteine residues. CXC chemokines (also called α chemokines) have the first two cysteine residues separated by a single AA (X) and typically attract neutrophils, T lymphocytes, B lymphocytes and natural killer cells. CC chemokines (or β chemokines), which account for the largest of chemokine subfamilies, don't have any interposed AA between their cysteine residues and are known to target mostly monocytes, macrophages, basophils, T lymphocytes and eosinophils, while having almost no effect on neutrophils. Chemokine CCL2 belongs to this family and will be further described in chapter 3.3.2. XC chemokines (or γ chemokines) contain just two cysteine residues and form a small family of only two known members, lymphotactin a and b (XCL1 and XCL2), which attract T lymphocytes. And lastly, the CX3C chemokine family (or δ chemokines) is represented by the only known member fractalkine (CX3C1) that has three amino acids between the first two cysteine residues and includes a mucin-like domain on its C-terminal (Rostene et al. 2007).

Chemokine cell-surface receptors are expressed by leukocytes and various non-hematopoietic cells. They are members of rhodopsin-like seven-transmembrane (7TM) receptor family and can be further divided in two groups: G protein-coupled chemokine receptors, which work via an activated Gi-type G protein, and atypical chemokine receptors that use protein arrestin instead of G proteins and act like chemokine scavengers, retaining their target chemokine and

causing it's decrease in the environment. In correspondence with their specific ligands, the receptors are designated CXCRn, CCRn, XCRn and CX3CRn (Bachelerie et al. 2014).

Chemokines play an important role in immune cells migration and their effects range from positioning and residing of immune cells during their development to their immediate attraction during an acute immune response (Griffith et al. 2014). In central nervous system, chemokines are involved in activation of residing microglia and astrocytes and they attract circulatory monocytes from the blood stream in case of immunological alert. There, chemokines can be produced by glial and neuronal cells that both can also express chemokine receptors, which indicates their mutual communication via chemokines (Conductier et al. 2010). However, in last years it became clear that chemokines also affect the signal transmission in neural synapses itself and that they might act here as important neuromodulators (Grace et al. 2014).

6.2. Spinal CCL2 in nociceptive transmission

6.2.1. Chemokine CCL2

CCL2, also called monocyte chemoattractant protein – 1 (MCP-1), is the first discovered human chemokine from the CC family and it was originally described thanks to its strong attractive effect on monocytes. It is encoded by a gene located on chromosome 17 (q11.2) and it is composed by 76 AA in total weight of 13kDa. The sequence homology between CCL2 and the rest of CCL chemokines is relatively high, from 61% for CCL4 and CCL8 to 71% for CCL7 (Van Coillie et al. 1999). Mutational analysis of CCL2 has revealed two regions of its primary structure that are crucial to its biological function – sequences of residues 10 – 13 and 34 – 35. As to its higher spatial structure, it turns out that CCL2 monomers, which are composed of four β sheets and 2 α helixes, are likely to form pocket-shaped dimers in natural solutions (Deshmane et al. 2009).

Production of CCL2 in central nervous system (CNS) was long time considered to be restricted to astrocytes, residing microglia and endothelial cells. It is also known to be released by infiltrated macrophages during local inflammation (Semple et al. 2010). However, it is now established that neuronal cells themselves can produce and release CCL2 in cerebral cortex and subcortical brain regions, such as hippocampus, substantia nigra and others (Banisadr et al. 2005), as well as on the level of the the spinal cord DH. Primary afferent neurons of DRG have been showed to constitutively express CCL2, to store it in secretory vesicles in their central terminals in the DH and to release it to the environment (Dansereau et al. 2008a; Van Steenwinckel et al. 2011).

Biological effects of CCL2 are mostly mediated through CCR2, a glycoprotein receptor coupled with a Gi protein. Although CCR2 can bind up to five CCLs (CCL2, CCL7, CCL8, CCL12 and CCL13), CCL2 remains the most potent ligand leading to the strongest activation of relevant signal pathways (Gouwy et al. 2004). There are two alternatively spliced forms of human CCR2, CCR2A and CCR2B, which each can stimulate distinct signalling pathways in the recipient cell. The dominant form is however CCR2B that represents about 90% of all CCR2s expressed on cellular surfaces (Van Coillie et al. 1999). CCL2 binds to CCR2 on multiple sites that are all required for initiation of G protein signalling, leading to activation of several intracellular signalling cascades, such as inducement of mitogen activated protein kinases (MAPKs), enhanced production of diacylglycerol (DAG) by protein kinase C (PKC) and release of calcium from storage vesicles through phospholipase C (PLC) (Old and Malcangio 2012).

CCR2 expression has been noted in various cell types residing in CNS, such as basophils and monocytes, as well as endothelial cells under inflammatory conditions (Yamasaki et al. 2012) and in human fetal astrocytes (Andjelkovic et al. 2002). There is some controversy about CCR2 expression in microglia, as there are several confirming works (Boddeke et al. 1999; Zhang et al. 2007) but also some disproving ones, for example CCR2 mRNA has not been found in cultured microglia (Zuurman et al. 2003) nor in acutely isolated microglia from adult mouse brain (Beutner et al. 2013; Hickman et al. 2013). Such discord in reported CCR2 expression could be possibly caused by detection of CCR2-positive inflammatory monocyte cells that migrate into the spinal cord and can be mistaken for microglia. There is however no doubt of CCR2 expression in neurons, which was detected both in specific regions of rat brain (Rostene et al. 2007) and in the spinal cord, where it is expressed in central terminals of DRG neurons and potentially also in secondary neurons of the superficial DH laminae (Gao et al. 2009; Jung et al. 2009).

6.2.2. Spinal CCL2 in normal and pathological nociception

Based on the expression patterns of CCL2 and CCR2, it has been many times suggested that CCL2 might serve as a communication medium between neighbouring neurons, as well as between neuronal and glial cells, with possible auto- and paracrine effect. Here we discuss the role CCL2 plays in cellular communication during nociceptive transmission and we concentrate on its effects at the level of the DRG and the DH of the spinal cord.

In his work from 2008, Dansereau et al. showed that CCL2 can alter nociceptive signalling in naive rats. Using immunohistochemical staining, they reported co-localization of CCL2 and TRPV1 in small and medium DRG neurons also expressing peptides related to nociception, such

as SP and CGRP (Dansereau et al. 2008b). More recently, Van Steenwinckel et al. described CCL2 localization in secretory vesicles in synaptic buttons of central terminals of C and Aδ fibers. Furthermore, CCL2 was also observed along microtubular structures in relevant primary axons, which indicates that CCL2 is transported from DRG cellular bodies to their central axon terminals where they are released (Van Steenwinckel et al. 2011). Its secretion in DRG neurons is known to be at some basal level constitutive, however it was documented that it increases substantially after high-threshold stimulation (Grace et al. 2014). Using *in vitro* superfusion on neurons isolated from DRG and DH of a naïve animal, Dansereau et al. described calcium-dependent CCL2 secretion from central terminals of DRG neurons following KCl- or capsaicin-induced depolarization (Dansereau et al. 2008a). Furthermore, xperiments *in vivo* showed that intrathecal CCL2 injection to control animals caused both thermal hyperalgesia and mechanical allodynia lasting up to four days (Dansereau et al. 2008b; Spicarova et al. 2014a; Thacker et al. 2009) and that these effects can be prevented by pre-treatment with CCR2 antagonist INCB3344 or with CCL2-antidote (Dansereau et al. 2008b; Thacker et al. 2009).

It has been also showed that CCL2 is usually upregulated in injured DRG neurons and after direct damage of peripheral afferents (Grace et al. 2014), suggesting the involvement in nociceptive signalling associated with peripheral neuropathy. So far, CCL2 involvement has been reported in works using several types of animal models of neuropathic pain, mostly affecting the sciatic nerve. In the SNI model in rats, the analysis of RNA expression in the spinal cord DH (areas of L4 and L5) revealed about 20 differentially expressed genes crucial for nociceptive activity, including CCL2 and CCR2 (Yang et al. 2014). Van Steenwinckel et al. used rats with experimentally induced CCI to prove that upregulated CCL2 expression occurs during neuropathies, along with upregulation of other pro-inflammatory markers and activation of extracellular signal-regulated kinase (ERK) pathway. They also showed that this increase can be minimized by intrathecal administration of the CCR2 antagonist INCB3344 (Van Steenwinckel et al. 2011). More recently, Zhu et al. used a rat model of lumbar disc herniation and apart from persistent hypersensitivity, they documented increased expression of CCL2 and CCR2 mRNA in the DRG and the spinal cord DH. In the DRG, CCL2 mRNA was mostly increased in small neurons and CCR2 mRNA in infiltrated macrophages, while in the DH CCL2 and CCR2 expression was enhanced both in astrocytes and neurons. In this case, intrathecal injection of CCR2 antagonist RS504393 also significantly attenuated herniation-induced mechanical allodynia (Zhu et al. 2014).

Another recent study concentrates on CCL2/CCR2 signalling in animals with CCI of the infraorbital nerve, a branch of the trigeminal nerve. Trigeminal ganglion is the equivalent of DRGs for the trigeminal nerve and peripheral injury of these nerve fibers leads to development of

trigeminal neuropathic pain. Higher levels of CCL2/CCR2 were observed even 14 days following the injury and CCL2-immunoreactivity increased in DG and TG tissues. In this case, not the post-operative but only the preventive INCB3344 treatment delayed the signs of mechanical allodynia and associated biochemical changes (Dauvergne et al. 2014).

It was proposed that CCL2 released from central terminals of DRG neurons also triggers the response of microglia in the DH that accompanies the development of neuropathic pain, such effect is however doubtful because of the lack of CCR2 expression in DH microglial population (Jung et al. 2007). On the other hand, CCL2 is known to affect endothelial cells, which respond by increasing the production of receptors and tethering proteins that facilitate infiltration of monocytes and T-cells to the CNS, where these can interact with microglia and astrocytes and therefore enhance the immune response, possibly causing alternations in neural activity (Grace et al. 2011).

6.3. Interactions of chemokine and opiate systems in nociception

As was indicated in the previous chapter, chemokines can act as mediators between immune, neuronal and glial cells under conditions associated with pathological pain and they are implicated in neural sensitization and hyperalgesia. In many cases, chemokines were showed to maintain a complex relationship with inner opioid system, and such cooperation is assumed to help regulate the development of some pain-related states, both physiological and pathological (Parsadaniantz et al. 2015). And against the first impression, chemokines and opioids do not play strictly opposite roles – in some cases chemokines were showed to contribute to pain attenuation and conversely, opioid use can induce pain syndromes, such as OIH (Parsadaniantz et al. 2015; Fletcher and Martinez 2014).

The chemokine-opioid crosstalk is enabled by the proximity of opioid and chemokine receptors and their ligands that are often found in the same tissue, often expressed by the same cells. The first site of chemokine-opioid interaction in the nociceptive pathway is naturally the periphery, where the sensory system cooperates strongly with the immune system. In the site of peripheral tissue or nerve damage, released chemokines attract macrophages and T-lymphocytes from the blood stream. These activated leukocytes were observed to produce endogenous opioid peptides that interact with opioid receptors in peripheral terminals of DRG neurons, which leads to a decreased excitability of sensory pathway. Furthermore, inflammation can cause increased expression of opioid receptors in DRG neurons and their subsequent transport to the peripheral

terminals, where their higher density strengthens the opioid-induced analgesia (Stein and Machelska 2011). Conversely, in some cases opioids were showed to boost peripheral inflammatory mechanisms by stimulating leukocyte chemotaxis and the expression of inflammatory chemokines and their receptors (Happel et al. 2008; Wetzel et al. 2000). Also, all peripheral effects of endogenous opioids on immune cells are often chemokine-specific. For example, pre-treatment of human neutrophils and monocytes with enkephalins have been showed to interfere with attractive signalling of chemokines CXCL8, CCL5, CCL3 and CCL2 but such a pre-treatment had no significant effect on the chemotaxis to CXCL12 (Grimm et al. 1998).

At the level of the spinal cord, chemokine and opioid receptors can be found both pre- and postsynaptically, and a subpopulation of sensory DRG neurons has been showed to co-express both chemokine and opioid receptors in their central terminals (Rivat et al. 2014). These neurons are capable to produce opioid peptides and relevant chemokines and following their release, these substances act in autocrine and paracrine manner in the environment of the DH. Secreted chemokines contribute to heighten nociception in several ways. They can induce the release of CGRP, SP, and glutamate from primary afferents and at the same time they take part in activation of secondary DH sensory neurons (Milligan et al. 2004; Reaux-Le Goazigo et al. 2012). The pro-nociceptive actions of chemokines are to some extent balanced by the effects of endogenous opioids that are released by neurons and activated interneurons. These opioids act both through presynaptic opioid receptoes where they cause the inhibition of SP, CGRP and glutamate release from primary afferent terminals, and through the hyperpolarization of DH neurons (Kardon et al. 2014).

There is also evidence that binding of opioids to their receptors might substantially influence the function of certain neighbour chemokine receptors in neuronal cells. After a population of rat cortical neurons expressing both CXCR4 and MOP-R was treated with morphine, an inhibition of CXCR4 function was observed that caused attenuation of CXCR4-mediated increase in ERK levels (Sengupta et al. 2009). Conversely, chemokines were observed to alter the function of opioid receptors. Stimulation of DRG neurons by pro-inflammatory chemokines (such as CCL3, CCL2, CCL5 and CXCL8) resulted in desensitization of MOP-R caused by its internalization and in the inability to elicit a proper cellular response to application of DAMGO (Zhang et al. 2004). The influence of chemokines was also demonstrated in electrophysiological experiments in rats. Neurons from various brain compartments including cingulate cortex, hippocampus, and periaqueductal grey co-express MOP-Rs together with chemokines CXCL12, CX3CL1 and their receptors, which suggest their possible interaction. The patch-clamp recordings supported this theory when morphine effects were prevented by chemokine pre-treatment (Heinisch et al. 2011).

Although the mutual influence of opioid and chemokine systems in neuronal cells is probably based on a wide range of molecular mechanisms, only a few hypothesis have emerged so far. Interestingly, it seems that neural opioid receptors might form hetero-dimers with certain chemokine receptors like it was previously described in immune cells (Suzuki et al. 2002) and brain glia (Burbassi et al. 2010). This idea is supported by their known co-localization in neuronal membranes, as it was showed with MOP-R and CXCR4 in the rat periaqueductal grey and DRG (Heinisch et al. 2014). Such receptor proximity would enable, amongst others, conformation-induced changes and it would also hugely facilitate another phenomenon – the heterologous desensitization. This term refers to a situation when two unrelated GPCRs are expressed in the same cell and the activation of one of them leads to the inhibition of another. GPCR receptor desensitization is in general mediated by their phosphorylation and therefore the heterologous desensitization depends mostly on the capacity of each receptor to stimulate protein kinases, which subsequently inhibits the opposite receptor (Steele et al. 2002). In case of nociceptive neurons, heterologous desensitization might partially explain the development of inflammation-induced hyperalgesia under conditions of increased chemokine levels in the periphery or in the spinal cord.

In clinical research, the opioid-chemokine interaction is suspected to play a role in development of undesirable states associated with opioid treatment –opioid tolerance and opioid-induced hyperalgesia. During chronic opioid treatment, just like during inflammation and in neuropathic states, the communication between neurons and glial cells is strengthen and the levels of inflammation-related neuromodulators are increased, including interleukins, CCL2 and CXCL1. In the spinal cord, interactions of these substances with neural receptors subsequently lead to sensitization of spinal neurons and their increased excitability (Sun et al. 2014; Zhao et al. 2012). The idea of glial involvement in opioid-induced pathologies is further supported by the effect of glial inhibitors. It was observed that administration of minocycline (a drug inhibiting glial activation and the release of chemokines) prevents the development of opioid tolerance in rodents both after systemic (Fukagawa et al. 2013) and intrathecal chronic morphine treatment (Cui et al. 2008).

Johnston and al. have originally showed that daily intrathecal injection of morphine induces production of spinal pro-inflammatory chemokine CX3CL1 that can afterwards contribute to development of opioid tolerance, hyperalgesia, and allodynia (Johnston et al. 2004). Later on, it was demonstrated that following morphine treatment another chemokine, CXCL12, is upregulated in sensory DRG neurons in the rat and they implied that this chemokine might be involved in the development of opioid-induced tactile hypersensitivity. Moreover, intraperitoneal administration of a specific CXCR4 antagonist completely reversed already developed OIH (Wilson et al. 2011).

Interestingly, a recent study of Zhao and al. connected the development of opioid-induced tolerance and hyperalgesia to the activity of chemokine CCL2 in the spinal cord of rats. After 7 days of morphine treatment (administered intrathecally), an increase of CCL2 immunoreactivity was observed in the spinal cord, mainly in the secondary spinal neurons. When pre-treated with an intrathecal injection of a CCL2-neutralizing antibody (anti-MCP-1), the subjects showed lesser opioid tolerance than controls. Furthermore, immunostaining with a microglial marker, OX-42, showed a substantial decrease in microglia activated by morphine treatment, which indicates that CCL2 plays a role in the microglia response to chronic opioid treatment (Zhao et al. 2012). Altogether, although a single study does not represent a sufficient proof of the CCL2 role in OIH development, it certainly suggests a possible new target in treatment of opioid-induced pathologies.

EXPERIMENTAL PART

7. AIMS OF WORK

The experiments presented in this thesis were designed and conducted under the guidance of members of the Department of Functional Morphology, Institute of Physiology, Czech Academy of Science). The aims are therefore closely related to the objectives of their research. The main part of research in this laboratory is focused on modulation of synaptic transmission in the nociceptive pathway, mostly in association with pathological painful states. The previous study published by the laboratory suggests that the chemokine CCL2 might act as a modulator of excitatory synaptic transmission between primary afferent terminals and secondary neurons in the superficial dorsal horn of the spinal cord, and that its effects are at least partially mediated by TRPV1 channels (Spicarova et al. 2014a). These findings have served as a starting point for the experimental design presented here.

The aim of this work was to further develop the topic of the role of CCL2 in pain transmission at the spinal cord level and to clarify its effects in relation to the activity of opioid receptors that have been also largely implicated in pain regulation. The research was focused on the first synapse of the nociceptive pathway, which is formed by central endings of primary afferent neurons and secondary neurons in the spinal cord dorsal horn. This first synapse represents an important modulatory site for the transmission of painful stimuli towards higher processing centres and as such it becomes a promising source of new targets in pain management. However, to be able to identify optimal therapeutic targets it is necessary to first understand the processes that underlie local signal transmission.

In this work, we aimed to find answers to following questions:

1. How does application of TRPV1 endogenous agonist OLDA influence frequency and amplitude of mEPSCs recorded from the secondary nociceptive neurons in animal model of neuropathic pain? Does peripheral neuropathy lead to increased responsiveness of TRPV1 to its endogenous agonists?

2. How does application of TRPV1 agonist OLDA influence frequency and amplitude of mEPSCs recorded from the secondary nociceptive neurons in animal model of neuropathic pain after incubation with CCL2? Does the incubation with CCL2 lead to sensitization of TRPV1 to its endogenous agonists?

3. How does the activation of spinal μ-opioid receptor by its selective agonist DAMGO modulate synaptic activity measured by recording of mEPSCs in nociceptive dorsal horn neurons in spinal cord slices from naïve rat?

4. How does the application of μ-opioid receptor agonist DAMGO modulate frequency and amplitude of mEPSCs recorded from the secondary nociceptive neurons treated with CCL2 solution? Does CCL2 have any modulatory effect on DAMGO-induced change in nociceptive synaptic activity?

8. METHODS

All experimental methods and procedures were approved by the local Institutional Animal Care and Use Committee and followed the guidelines of the IASP, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, and the European Communities Council Directive of 24 November 1986 (86/609/EEC). All experiments were consistent with the Animal Protection Act of Czech Republic 246/1992 Sb. and were designed to minimize animal suffering while using as few animals as possible.

The whole research is based on electrophysiological recordings of excitatory postsynaptic currents (EPSC) in superficial neurons in the dorsal horn of the spinal cord. These recordings were obtained using the patch-clamp method in the whole-cell mode on target cells in acute slices of the lumbar section of the spinal cord in rat.

Substance	Chemical formula
Agarose	$(C_{12}H_{18}O_9)_n$
Bicuculline methiodide	$C_{21}H_{20}INO_6$
Bovine serum albumin (BSA)	-
Calcium chloride	$CaCl_2$
Capsaicin	$C_{18}H_{27}NO_3$
Carbogen	O_2 (95%) + CO_2 (5%)
CCL2	-
Cesium hydroxide	CsOH
Cesium chloride	CsCl
D-glucose	$C_6H_{12}O_6$
[D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO)	$C_{26}H_{35}N_5O_6$
dimethyl sulfoxide (DMSO)	C_2H_6OS
EGTA	$C_{14}H_{24}N_2O_{10}$
Gluconate delta lactone	$C_6H_{10}O_6$
HEPES	$C_8H_{18}N_2O_4S$
Magnesium chloride	$MgCl_2$
N-Oleoyldopamine (OLDA)	$C_{26}H_{43}NO_3$
Potassium dihydrogen phosphate	KH_2PO_4
Potassium chloride	KCl
Sacharose	$C_{12}H_{22}O_{11}$
Sodium bicarbonate	$NaHCO_3$
Sodium chloride	NaCl
Sodium phosphate monobasic	NaH_2PO_4
Strychnine hydrochloride	$C_{21}H_{22}N_2O_2$
Tetrodotoxin citrate (TTX)	$C_{11}H_{17}N_3O_8$

Table 8.1: All used substances and their chemical formulas. Adapted and modified from master thesis of P. Adámek (Adámek 2014)

8.1. Chemicals used in the experiments

All chemical substances used during the experiments are listed in Table 8.1. The original chemicals were purchased from supplier companies Sigma-Aldrich (USA), Tocris Bioscience (Great Britain), R&D Systems, Inc. (USA), Linde Gas a.s. (Czech Republic) and Abbott Laboratories Ltd (Great Britain).

Two types of extracellular solution (ECS) were used in the course of preparation of spinal cord slices (for detailed description see Chapter 8.3). During the acquisition of slices and the short incubation that followed, the sample tissue was kept in the dissection ECS with low concentration of calcium ions. Ready slices were then transferred into the recording ECS with normal concentration of calcium ions, in which they were further incubated and subsequently measured. Both solutions were continuously supplied with carbogen (a mixture of carbon dioxide (5%) and oxygen gas (95%)) in order to preserve the optimal pH and sufficient oxygen saturation of the environment. The exact compositions of dissection and recording ECS are showed in Table 8.2.

For the recording, glass microelectrodes were filled with intracellular solution (ICS) as it is further described in chapter 8.4. CsOH was used to regulate the pH of the solution to the required value 7,2. The composition of the ICS is showed in Table 8.2.

When cutting the spinal cord slices, the lumbar section of the spinal cord was held steady in the vertical position by two blocks of agar jelly. This jelly had been prepared prior to the experiment from heated agar solution that was poured into a petri dish and after solidifying kept in the fridge. The composition of the agar solution is showed in Table 8.2.

During the patch-clamp recording the conditioning and tested chemical substances were applied in form of solutions that had been prepared by diluting very concentrated original stock solutions. Capsaicin and OLDA were dissolved in DMSO with resulting content of DMSO not higher than 0,1% of the total volume, while CCL2 was dissolved in 0,1% BSA and all the other substances in re-distilled water.

Solution	Components and their concentration (mM)	Osmolarity (mOsm)
Dissection ECS	NaCl (95); KCl (1,8); $MgSO_4$ (7); $CaCl_2$ (0,5); KH_2PO_4 (1,2); $NaHCO_3$ (26); D-glucose (25); sacharose (50)	290
Recording ECS	NaCl (127); KCl (1,8); $MgSO_4$ (1,3); $CaCl_2$ (2,4); KH_2PO_4 (1,2); $NaHCO_3$ (26); D-glucose (25)	290
ICS	Gluconate delta lactone (125); CsCl (15); CaCl2 (1); EGTA (10); HEPES (10); Na_2ATP (2); NaGTP (0,5); CsOH	310
Agar solution	NaCl (160); KCl (2,5); $MgCl_2$ (5); $CaCl_2$ (0,5); D-glucose (10); HEPES (10)	-

Table 8.2: Used solutions and their compositions. Adapted from the master thesis of P. Adámek (Adámek 2014)

8.2. Experimental animals

8.2.1. Naïve animals

Male Wistar rats were used in all the experiments. The animals were acquired from the breeding station at the Institution of Physiology (Czech Academy of Sciences, Vídeňská 1083 Prague 4) where they were held in plastic cages with soft bedding, allowed free access to food and water, and maintained on a 12 h light, 12 h dark cycle.

8.2.2. Animal model of peripheral neuropathy

In our experiments we used the model of chronic constriction injury (CCI) that consists of multiple loose ligation of *n. ischiadicus* leading to chronic nerve oppression, which consequently caused nerve swelling and degradation (Gabay and Tal 2004). This step of experimental work was performed by Pavel Adámek, a PhD student in our laboratory.

Male Wistar rats were operated within P19 – P23. First, the animal was deeply anesthetized with 3% isoflurane (Forane, Abbott Laboratories Ltd), fixed to an operating pad and throughout the whole operation supplied with the isoflurane mixture through a customized breathing tube to maintain the anesthesia. On the dorsal side of both thighs, the fur was removed and the relevant patch of skin was disinfected with 3% iodine alcohol tincture (Jodisol Spray, SpofaDental a.s.). Bilaterally, *n. ischiadicus* was revealed through a cut in *m. biceps femoris* and 3 loose successive ligatures of non-absorbable surgical silk, approximately 1mm apart were applied. After the ligation was complete, the operation would was closed and disinfected.

Operated animals were kept in separately in plastic cages with soft bedding and free access to water and food. The animals were tested for the presence of increased responsiveness to mechanical stimuli and used for electrophysiological experiments five days later as described in Chapter 8.3.

8.3. Preparation of acute spinal cord slices

The protocol for spinal cord slices preparation didn't vary between naive rats and animals with peripheral neuropathy. The naive animals were processed within postnatal days P19 – P23, whereas the rats with neuropathy were on average 5 days older (P24 – P28). For the preparation of acute spinal cord slices, the rats were anesthetized with 3% isoflurane (Forane, Abbott Laboratories Ltd) and after laminectomy, the spinal cord was extracted. Immediately after securing the sample tissue, the anesthetized rats were euthanized by disruption of medulla and anesthetic overdose.

The lumbar part of the spinal cord was excised and the tissue was immediately immersed in dissection ECS. The ECS was cooled to the temperature under 4°C in order to slow down the cellular metabolism and prevent any damage caused by ischemia. Ventral and dorsal roots of the spinal cord were cut away and spinal meninges were carefully removed while making sure the superficial layers of the dorsal horn were not disturbed. A section of approximately 1 cm of the lumbar part was set in the vertical position in a groove between two agar blocks prepared beforehand. The blocks were then fixed with superglue on the holder pad of an automatic vibrating blade microtome Leica VT1200 S (Leica Biosystems Nussloch GmbH, Germany) and immersed in ice cold bath of dissection ECS (< 4°C). 10 - 15 transversal slices of 300 μm were cut with a razor or a sapphire blade.

Finished slices were progressively moved into an incubation chamber filled with dissection ECS that had been warmed up to 35°C in water bath Memmert WB-7 (Memmert GmbH, Germany). The slices were incubated there for 30 mins and then moved to another incubation chamber with recording ECS at room temperature (21 – 25°C), where they were incubated for at least 2 hours before they were used for the experiment.

According to our observations, the viability of target neurons in the sample tissue strongly correlated with the speed of the preparation of slices. In an ideal case, we were able to complete the first incubation within an hour from the set off of the anesthesia. However, the quality of the sample is also dependent on precise, careful handling because any oppression of the nerve tissue represents a potential cause of cellular degradation.

8.4. Electrophysiological recordings

8.4.1. Technical equipment

Sample neurons in the spinal cord slices were visualized with differential interference contrast (DIC) microscope (Leica, DM LFSA, Germany) and the images were captured by a near infrared-sensitive camera (Hitachi KP-200P, Japan). NIR-LED diode with the wavelength of 850 nm was used as a source of infra-red light. The image was processed through software PixeLINK® Capture OEM 7.25 and displayed on a standard TV/video monitor. Electrical currents were recorded with patch clamp amplifier Axopatch 1D (Axon Instruments, Inc., USA) and analog-digital converter Axon CNS Digidata 1440A (Molecular Devices, LLC., USA). The recordings were processed by program Clampex 10.2 and program Clampfit 10.2 was used for later analysis.

Both programs are included in a software package pClamp version 10.2 (Axon Instruments, Inc., USA).

8.4.2. Preparation of glass microelectrodes

A fresh batch of glass microelectrodes was prepared before each experiment. Borosilicate glass tubes (diameter mm; Rückl Glass a.s., Czech Republic) were heated and pulled at a pipette puller model P-97 (Sutter Instrument Co., USA). Ready microelectrodes were filled with ICS through a plastic tip for loading capillaries (Eppendorf Czech & Slovakia s.r.o, Czech Republic). In the experiments I only used microelectrodes that had resistance between 3,5 and 6,0 MΩ.

8.4.3. Patch-clamp recording

At the beginning of the experiment, a slice of lumbar spinal cord was placed in a small recording chamber, where it was held in place with a customized nylon fiber grid spun inside of a small platinum frame. A membrane pump was used to generate positive pressure inside the recording microelectrode ensuring a clean tip of the pipette, while searching for a neuron in the slice. All the recordings were made in a whole cell patch-clamp mode with the neurons clamped at -70 mV. The series resistance of neurons was routinely compensated by 80 % and was monitored during whole experiment. Cells with a series resistance >20 MΩ were not recorded. The recording started approximately 3 minutes after the whole cell connection was established, when the neuronal activity stabilized. In this work, we recorded spontaneous and miniature excitatory postsynaptic currents (sEPSCs, mEPSCs) in neurons located in superficial laminae I and IIo in the spinal cord dorsal horn (see Figure 8.1).

8.4.4. Application of chemicals and recording protocols

In the course of electrophysiological recording, the recording chamber containing a spinal cord slice was irrigated with ECS that was saturated with carbogen (O2 (95%) + CO2 (5%)) and progressively pumped away to keep the flow stable (approximately 2 ml/min). This system allowed a sufficiently fast exchange of applied solutions within the recording tank.

For the recording several inhibitors were added to the ECS: bicuculline (10 μM; $GABA_A$ receptor reversible antagonist) and strychnine (5 μM; glycine and acetylcholine receptors competitive antagonist). In order to record miniature EPSC (mEPSC), tetrodotoxin (TTX) was added (0.5 μM; voltage-gated sodium channels block) that ensured the inhibition of spontaneously generated action potentials.

In the first part of this work, the protocol was similar for both measured groups of neurons and comprised of successive application of ECS without TTX (recording of sEPSCs), ECS with added TTX (recording of mEPSCs) and ECS with TTX and TRPV1 agonist OLDA (0.2 μM). All 3 intervals were 4 mins long.

In the second part of this work, MOP-R specific agonist DAMGO (1 μM) and chemokine CCL2 (10 nM) were applied. The recording protocol for the group incubated with CCL2 ('CCL2 incubation') and the control neurons ('Control') comprised of a control interval ('CTRL', 4 mins) when only the recording ECS with inhibitors was flowing in (the ECS contained TTX, only mEPSCs were recorded). Then the ECS with DAMGO was applied ('DAMGO', 3 mins) and this interval was followed by a wash-out period ('Wash-out', 25 mins). The recording protocol for the group treated with acute CCL2 application ('Acute CCL2') was similar to the protocol described above but was extended by a 4 mins interval preceding the application of DAMGO, when the ECS with CCL2 flowed in. The CCL2 application continued simultaneously with the DAMGO application (altogether for 7 mins) and was terminated before the wash-out period.

In the very end of every recording in both parts of the work, TRPV1 agonist capsaicin ('CAPS', 0.2 μM) was applied in order to verify the presence of TRPV1 channels in primary nociceptive afferents. Neurons with capsaicin-sensitive primary afferent input were identified by an increase of EPSC frequency (>20%) following CAPS administration.

8.5. Data analysis

Program Clampfit 10.2 from the software pack pClamp 10.2 was used for off-line analysis of the obtained data. From every measured interval of a recording, a section of the last 2 mins was analyzed and the same parameters were applied in all the assessed recordings. The whole cell responses were first low-pass filtered at 2 kHz and digitally sampled at 10 kHz. Individual events and corresponding amplitudes were then manually registered with the amplitude threshold set at 6pA (= at least twice the level of the noise).

Final frequency and amplitude of EPSCs were averaged and are showed either in absolute values (average Hz and pA) or were standardized to the CTRL section of the recording (%) and are complemented with the value of the standard error of mean (SEM).

Statistical analysis and graph generation were done in program SigmaPlot 12.3. One-way ANOVA test was used to assess the difference in EPSC frequency and amplitude amongst several groups of data, whereas a simple t-test was used for comparison of only two groups. To test any

statistically relevant changes between subsequent measurements from the same recording, one-way ANOVA repeated measures test was used, followed by a post hoc test (Student-Newman-Keuls, Bonferroni). In all analytical tests, the $P<0.05$ was considered statistically significant.

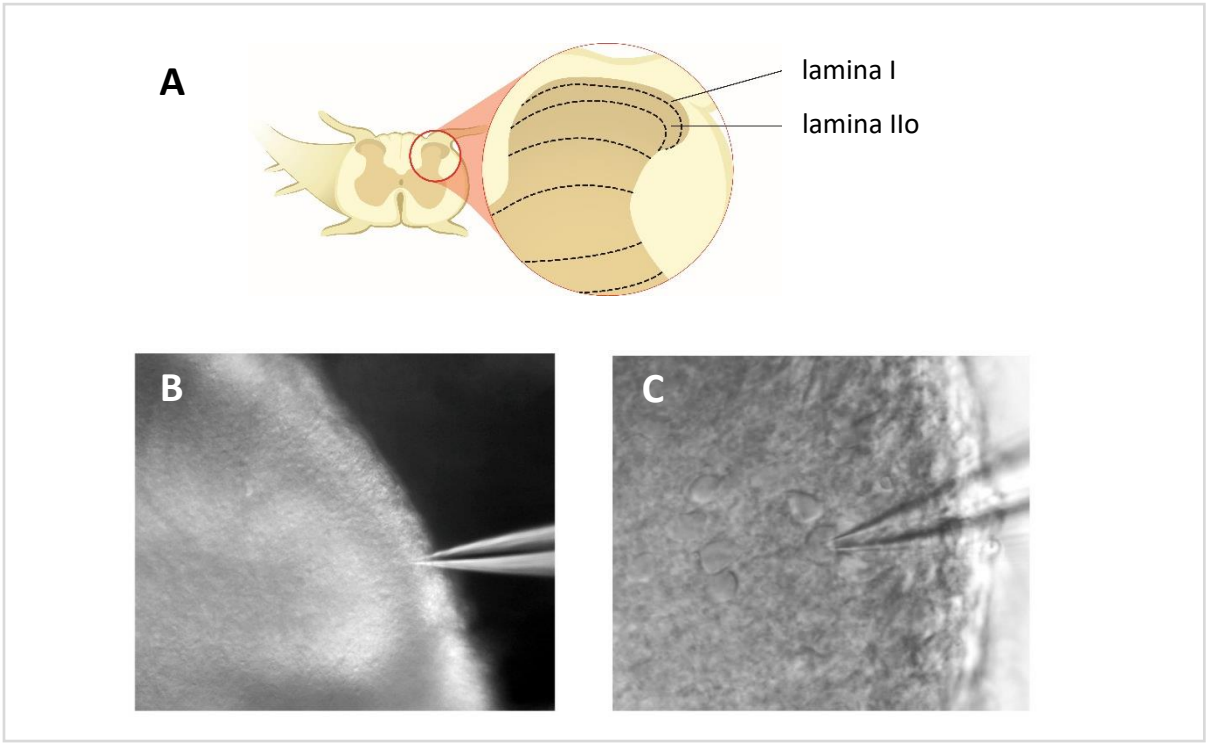

Figure 8.1: Patch-clamp recordings were made in neurons located in superficial laminae of the spinal cord dorsal horn. (**A**) Schematic localization of targeted laminae I and IIo within the dorsal horn. (**B, C**) Photographic documentation of the microelectrode positioning. Both pictures were adopted with permission from archives of the Department of Functional Morphology, Institute of Physiology, Czech Academy of Science

9. RESULTS

9.1. Modulatory effects of CCL2 on synaptic activity and TRPV1 activation by its endogenous agonist OLDA in superficial spinal cord dorsal horn during peripheral neuropathy (CCI)

In a previous study published by our laboratory (Spicarova and Palecek 2009), it was showed that a solution with low concentration of OLDA (0.2 μM) did not evoke any change in the miniature EPSCs (mEPSC) frequency in neurons from naïve rats and that much higher concentration of OLDA (10 μM) was needed to induce a frequency increase. This increase was blocked by TRPV1 antagonists, showing that the effect of OLDA is directly mediated by TRPV1. Furthermore, in spinal cord slices from animals with peripheral inflammation, already the application of a low concentration of OLDA evoked an increase in mEPSC frequency, suggesting that peripheral inflammation might cause sensitization of TRPV1.

The aim of the experiment reported here was to verify that similar sensitization of TRPV1 occurs also in the animal model of peripheral neuropathy and to describe the effect of additional incubation with CCL2 on the activity of the first nociceptive synapse. Animals with induced CCI (5 days after the operation, P24 – P28) were used for the preparation of lumbar spinal cord slices. To measure the activity of the first nociceptive synapse, electrophysiological recordings were made from 23 neurons located in superficial layers of the dorsal horn (laminae I and IIo). The activity of synaptic transmission was characterized using recordings of mEPSC.

The experiment was conducted on two groups of neuronal cells subjected to distinct conditions. The control group (12 neurons) was incubated in the recording ECS with no additives, whereas the second group (11 neurons) was incubated in the recording ECS enriched with CCL2 (10 nM). Patch-clamp recordings were made at room temperature ~ 24 °C. The recording protocol was the same for both groups of neurons and comprised successive application of ECS without TTX (in order to record sEPSCs), ECS with TTX (in order to record mEPSCs) and ECS with TTX and OLDA (0.2 μM).

9.1.1. Application of low concentration TRPV1 agonist OLDA increased mEPSC frequency during peripheral neuropathy.

In the control group 5 days after the neuropathy induction, acute application of TRPV1 agonist OLDA in low concentration (0.2 μM) led to a significant increase ($P<0.05$) of mEPSC frequency in neurons in the superficial layers of the spinal cord dorsal horn. The average frequency

of mEPSCs grew up to 120.6 ± 9.2 % in comparison with mEPSC frequency before the application (Fig. 9.1A). To illustrate the effect of OLDA application, Figure 9.1B shows representative excerpts from a single recording before and after the application of OLDA.

Neurons in the control group did not react to OLDA homogenously. From all 12 cells, in 10 we observed an increase in mEPSC frequency, whereas the remaining 2 reacted by a slight decrease in mEPSC frequency (on average to 85.8 %).

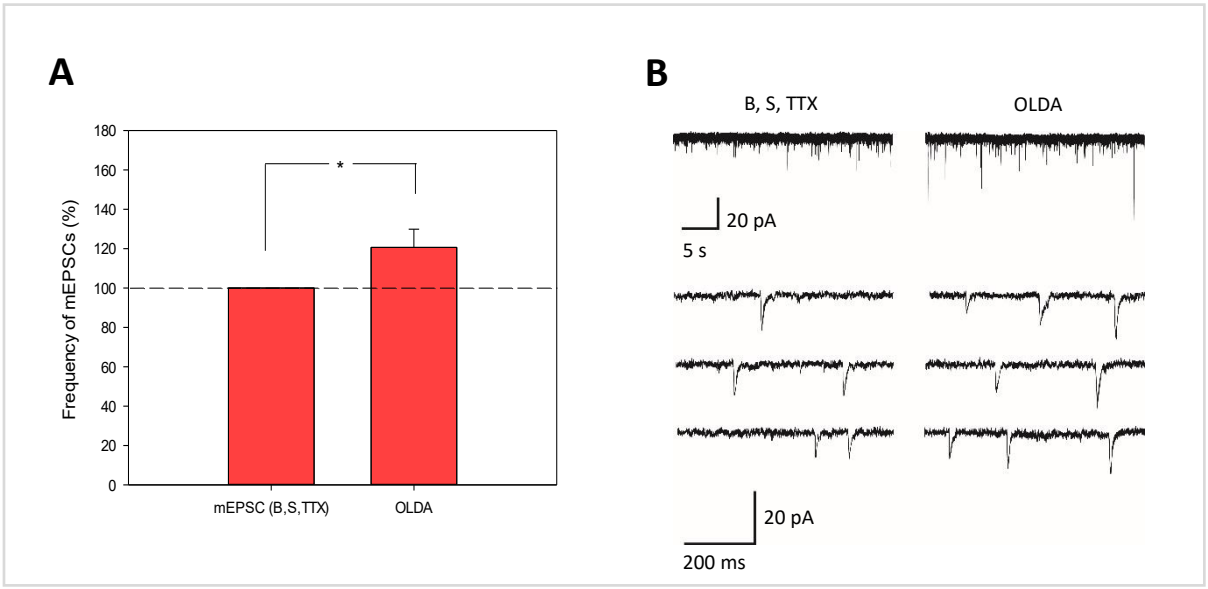

Figure 9.1: TRPV1 agonist OLDA in low concentration caused an increase in mEPSC frequency in animal model of peripheral neuropathy. **(A)** In comparison to the prior interval (B,S,TTX 100 %), an acute application of OLDA (0.2 µM) led to an average increase in mEPSC frequency to 120.6 ± 9.2 % (n=12, * P<0,05). **(B)** Excerpts from a single recording show an increase of mEPSC frequency after the application of OLDA (0.2 µM). Upper images display intervals ~30 s long, lower images represent close-ups from upper intervals in length of ~600 ms.

9.1.2. In neurons incubated with CCL2, application of low concentration of OLDA increased mEPSC frequency with variable onset.

Amongst neurons that were incubated in ECS with CCL2 (10 nM), acute application of OLDA (0.2 µM) also led to an increase of mEPSC frequency but we observed a great variability in response time amongst the individual neurons. Using the data from a rigidly set interval similar to the control group (3. – 4. min), the average increase in mEPSC frequency was 132.8 ± 21.8 % when compared to the average mEPSC frequency of the preceding interval (B,S,TTX 100%). In this case the increase did not show any statistical significance. However, when we analyzed the intervals of maximum activity, the average frequency of mEPSCs grew to 160.5 ± 26.2 % in comparison with

mEPSC frequency before the application of OLDA and this increase was significant (P<0,05; see Figure 9.2A).

When comparing the data obtained from controls and incubated neurons, statistical testing did not show any significant difference, although the average increase in mEPSC frequency appears much higher in neurons incubated with CCL2 (considering the values from maximum frequency intervals). This is probably caused by a very broad range of values measured amongst the incubated neurons, while in control neurons the individual values were less variable (see Figure 9.2B)

In the group incubated with CCL2, the recorded neurons also didn't react homogenously to the OLDA application. From 11 recorded cells, in 9 we observed an increase in mEPSC frequency, whereas the remaining 2 reacted by a slight decrease in mEPSC frequency (in average to 71.8 %).

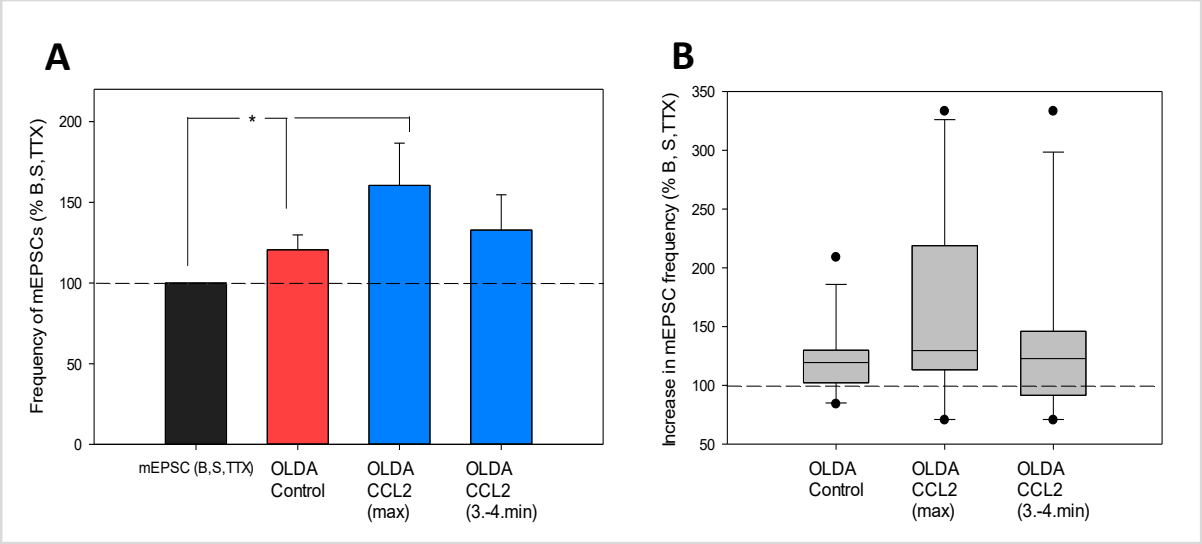

Figure 9.2: In neurons previously incubated with CCL2 (10 nM), TRPV1 agonist OLDA in low concentrations (0.2 μM) caused an increase in mEPSC frequency with a variable onset across the population of the recorded cells. **(A)** Average mEPSC frequency measured within 3. – 4. min in incubated neurons increased to 132.8 ± 21.8 % (P>0.05; paired t-test) when compared to the average mEPSC frequency of the preceding interval (B,S,TTX 100%). The data from intervals of maximum activity in incubated neurons showed an average increase to 160.5 ± 26.2 % (P<0.05; paired t-test). Statistical comparison (t-test) showed no significant difference between data obtained from the control group and data from neurons incubated with CCL2. **(B)** Neurons previously incubated with CCL2 showed much broader range of responses following application of OLDA. For illustration we show the distribution of values of mEPSC frequency measured within the OLDA interval both in the control and incubated neurons ('CCL2')

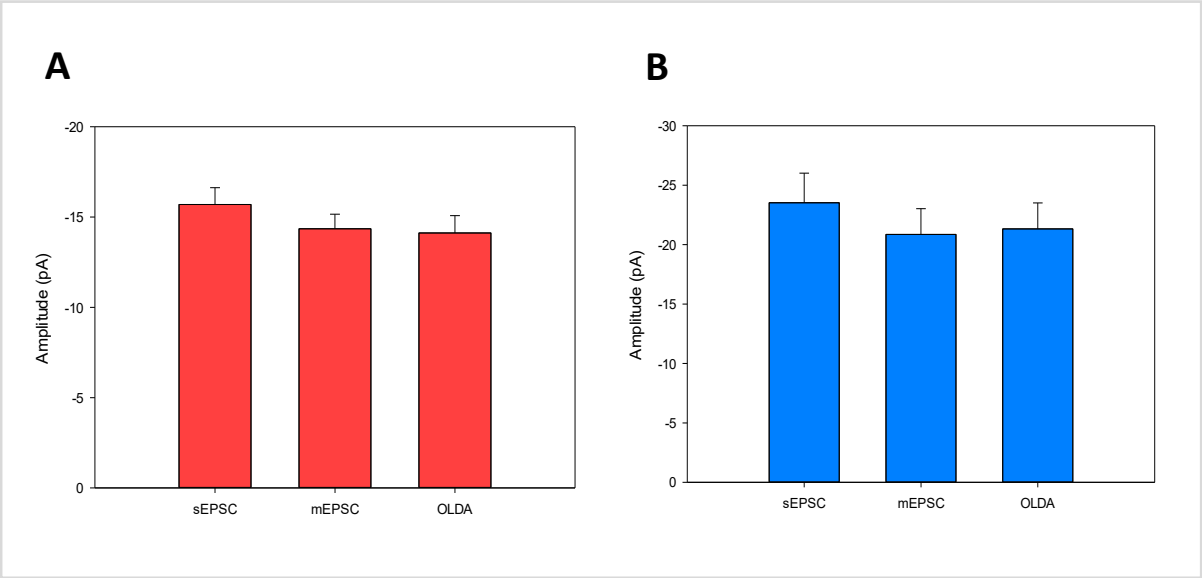

Figure 9.3: In both measured groups, the average amplitude didn't significantly change in the course of recording. **(A)** In the control group of neurons (n=12) the average amplitude was 15.7 ± 0.9 pA for sEPSC, 14.4 ± 0.8 pA for mEPSC and 14.1 ± 1.0 pA following the application of OLDA (0.2 μM). None of the changes were statistically different (P>0.05; paired t-test). **(B)** In the group of neurons that were incubated with CCL2 (n=11) the average amplitude was 23.5 ± 2.5 pA for sEPSC, 20.9 ± 2.2 pA for mEPSC and 21.3 ± 2.2 pA following the application of OLDA (0.2 μM). None of the changes were statistically different (P>0.05; paired t-test).

9.1.3. Under the conditions of peripheral neuropathy, the application of TRPV1 agonist OLDA didn't have any effect on the average mEPSC amplitude.

In the control group of neurons, the average EPSC amplitude progressively slightly decreased in the course of the recording and I didn't observe any change in amplitude related to the application of OLDA (0.2 μM). The average amplitude of sEPCS was 15.7 ± 0.9 pA, after the application of TTX it dropped to 14.4 ± 0.8 pA and it was stable at 14.1 ± 1.0 pA following the application of OLDA (P>0.05; see Figure 9.3A).

In neurons treated with CCL2, the average mEPSC amplitude showed a slight increase following the application of OLDA. The average amplitude of sEPCS was 23.5 ± 2.5 pA, it decreased to 20.9 ± 2.2 pA after the application of TTX, and the average amplitude of mEPSC after the application of OLDA was 21.3 ± 2.2 pA. None of these changes were statistically significant (P>0.05; see Figure 9.3B).

9.1.4. Under the conditions of peripheral neuropathy, the incubation of spinal cord slices with CCL2 increased basal synaptic activity.

In neurons that were previously incubated with CCL2 (10 nM), we observed higher basal frequency of sEPSC and mEPSC in comparison with the control group. The average sEPSC frequency was 3.85 ± 1.10 Hz, while in controls it was 2.25 ± 0.42 Hz. Similarly, the average mEPSC frequency was 2.69 ± 0.78 Hz, while in controls it was 1.80 ± 0.28 Hz. However, the differences in EPSC frequencies were not statistically significant, probably due to low number of recoded neurons and relatively high spread of the measured values (see Figure 9.4A).

The amplitudes of sEPSC and mEPSC showed statistical significant differences between the control and CCL2 treated groups ($P<0.05$). In our recordings, the average sEPSC amplitude in cells previously treated with CCL2 was 23.5 ± 2.5 pA, whereas the average amplitude recorded in the control cells corresponded to 15.7 ± 0.9 pA. Similarly, the average mEPSC amplitude in the CCL2 incubated cells was 20.9 ± 2.2 pA while in control cells it was 14.4 ± 0.8 pA (see Figure 9.4B).

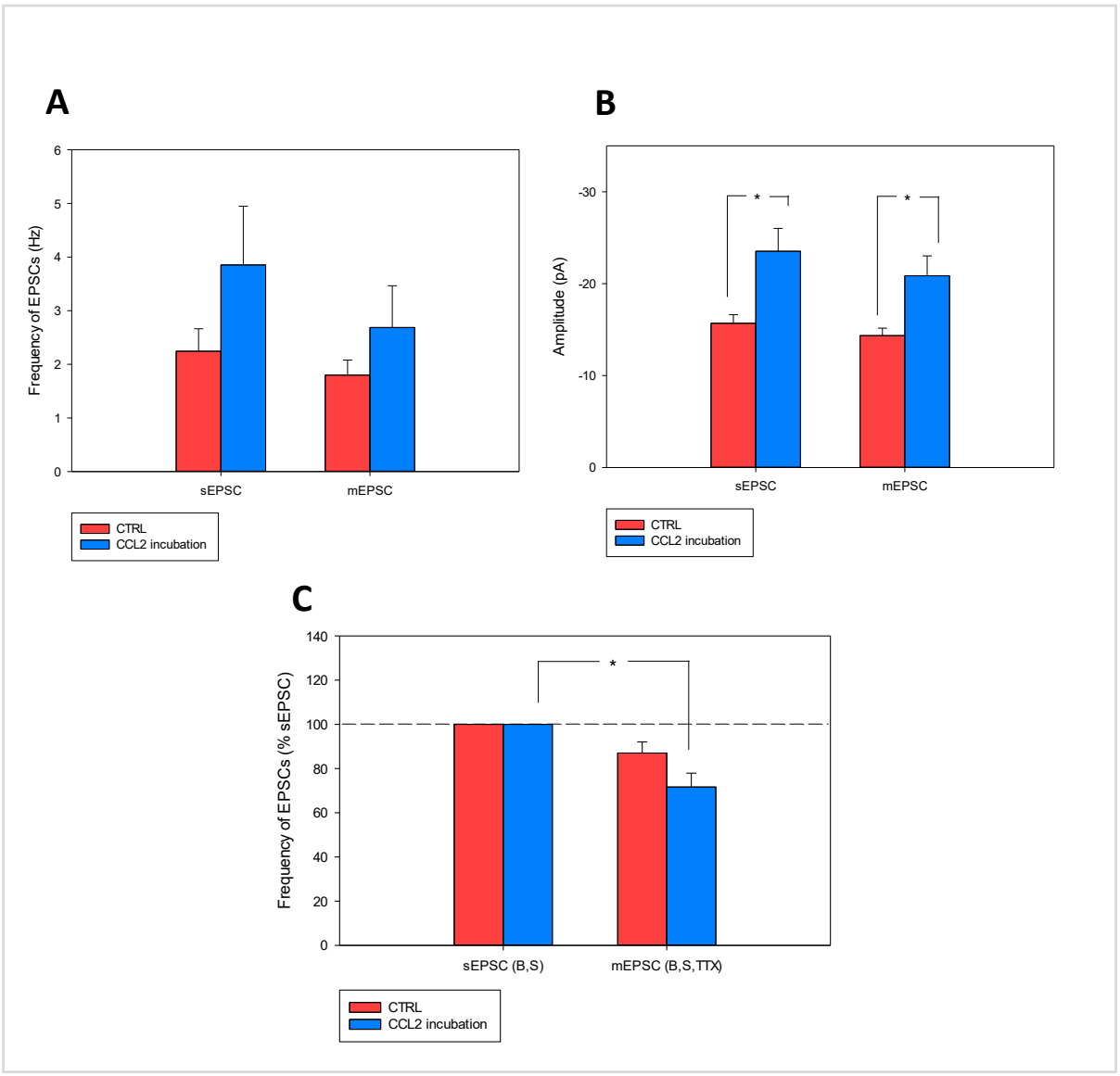

Figure 9.4: Incubation with CCL2 (10 nM) led changes in frequency and amplitude of mEPSC and sEPSC in superficial DH neurons from an animal model of peripheral neuropathy. **(A)** Average mEPSC and sEPSC frequency in control neurons was lower than in the incubated neurons. Statistical analysis did not show any significant difference between both tested groups ($P>0.05$; t-test). **(B)** Average mEPSC and sEPSC amplitude in control neurons was lower than in incubated neurons When the measured groups were compared against each other, the average amplitude of sEPSC and mEPSC is significantly higher after the CCL2 incubation ($P<0.05$; t-test). **(C)** In both tested groups the EPSC frequency diminished after the application of TTX. In the control neurons the decrease was to 87.5 ± 5.0 % sEPSC ($P>0.05$), whereas in cells incubated with CCL2 the decrease was to 71.6 ± 6.3 % sEPSC with $P<0.05$.

9.2. Modulatory effects of CCL2 on synaptic activity elicited by μ-opioid receptor agonist DAMGO application in nociceptive spinal cord neurons

In the second block of experiments, only naïve rats were used for the preparation of lumbar spinal cord slices. Electrophysiological recordings were made from 35 neurons located in superficial layers of the dorsal horn (laminae I and IIo) in order to describe the activity of the first nociceptive synapse. During all experiments, recording ECS enriched with TTX (0.5 μM) was used to block voltage-sensitive Na channels and to suppress spontaneous generation of action potentials, so only miniature EPSCs (mEPSCs) were recorded.

The modulatory effect of CCL2 was studied in two separate groups of neurons. In the first group (n=12), the spinal cord slices were incubated with CCL2 (10 nM) for 2 hours prior the electrophysiological recording. The slices in the second group (n=10) were incubated in recording ECS without any additive and CCL2 was applied acutely in the course of recording, therefore the protocol was by 4 mins longer (see Methods, Chapter 8.4.5). The data collected from the CCL2 treated neurons were compared to the data from the control neuronal cells (n=13) that were incubated in pure recording ECS and recorded using the shorter protocol without CCL2 application.

9.2.1. In control neurons, acute application of DAMGO decreased mEPSC frequency

In recordings from control neurons, we confirmed the effects of DAMGO that had been already reported in previous studies. In the interval during the application of DAMGO (1 μM, 3 min), the frequency of mEPSCs decreased on average to 66.9 ± 4.6 % when compared to the control section of the recording (CTRL 100 %; $P<0.001$, Fig. 9.5A). The mEPSC frequency was still lower during the last wash-out interval (wash-out minutes 23. – 25.), when the average mEPSC frequency represented 77.2 ± 13.4 % (CTRL 100%; $P<0.05$). To illustrate the acute effect of DAMGO, Figure 9.5C shows representative excerpts from a single recording in intervals before ('CTRL') and immediately after ('DAMGO') its application.

The immediate reaction to acute application of DAMGO was homogenous across the whole measured population of neurons (n=13). However, we observed variation in the further development of mEPSC frequency. From all the 13 measured cells, 7 showed a persistent decrease in mEPSC frequency, whereas in 4 cells we noted an increase above 100% CTRL towards the end of the wash-out period. The remaining 2 cells reacted by a transient increase of mEPSC frequency above 100% CTRL during the first wash-out interval (measured within wash-out minutes 3. – 5.) that was followed by a stable decrease below 100% CTRL in subsequent intervals.

When we pooled the data from wash-out and DAMGO sections into longer intervals (2 + 2 mins), we found statistically significant increase in later 2 intervals in comparison with the DAMGO/Wash 05 interval (P<0.05, see Figure 9.5B). That indicates that the mEPSC frequency slowly restores to its original values.

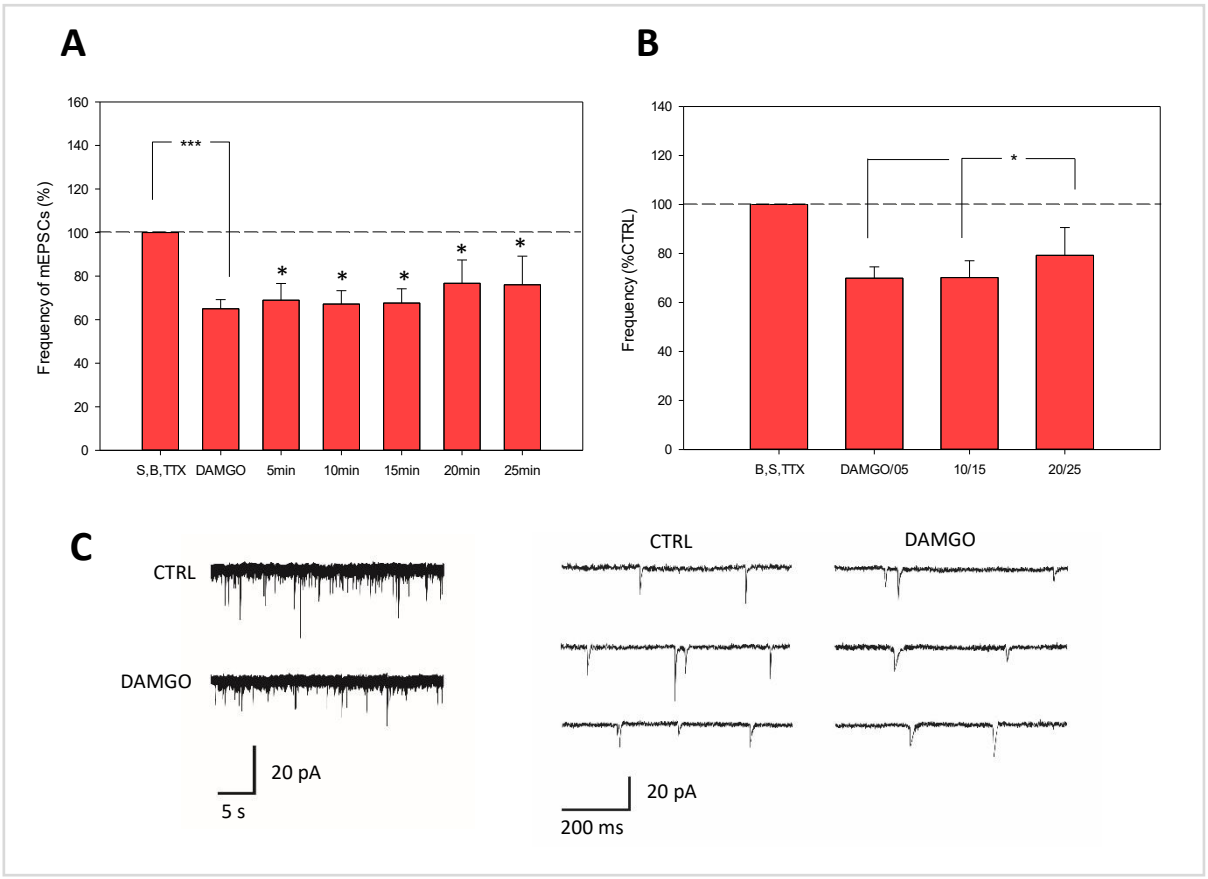

Figure 9.5: Application of MOP-R agonist DAMGO (1 µM) caused fast and persistent decrease in mEPSC frequency in neurons of the control group. **(A)** Within 2 minutes after the beginning of DAMGO application, mEPSC frequency decreased to 66.9 ± 4.6 % of the control section (CTRL 100 %; *** P<0.001; RM ANOVA on Ranks and Student-Newman-Keuls test, paired t-test). During the following 25 mins of wash-out, mEPSC frequency remained significantly lower than the original value of the CTRL interval (CTRL 100 %; * P<0.05; RM ANOVA on Ranks and Student-Newman-Keuls test). **(B)** Slow restoration of mEPSC frequency can be seen in growing average values of pooled intervals. The last wash-out interval already shows a significant increase in mEPSC frequency in comparison to previous two wash-out intervals (* P<0.05; RM ANOVA on Ranks and Student-Newman-Keuls test) **(C)** Excerpts from a single recording show an increase in mEPSC frequency after the application of DAMGO (1 µM). Images at the left side display intervals ~30 s long, while right side images represent close-ups from upper intervals in length of ~600 ms.

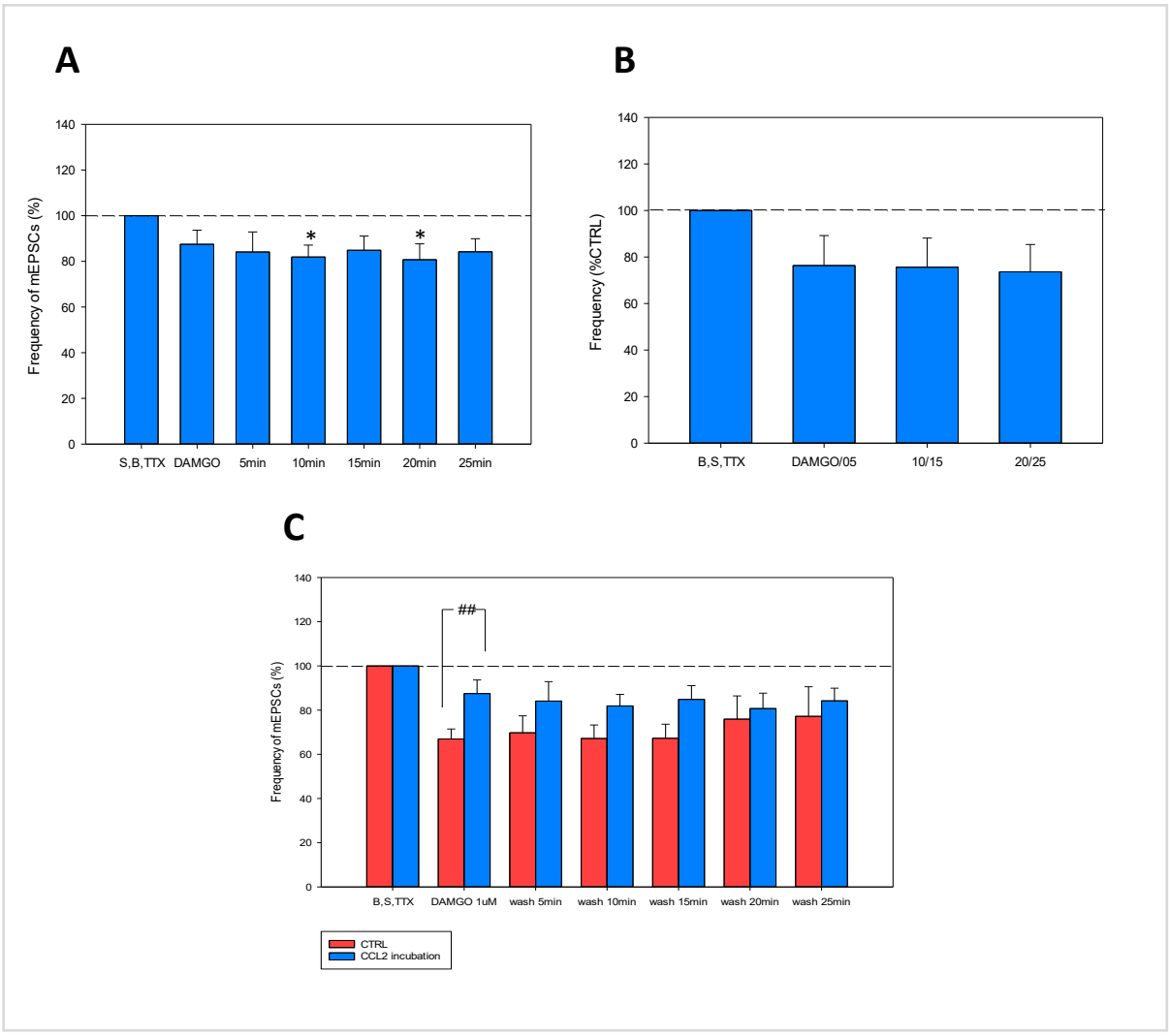

Figure 9.6: In neurons previously incubated with CCL2 (10nM), the inhibitory effect of MOP-R agonist DAMGO (1 μM) was partially suppressed and only a moderate decrease in mEPSC frequency was observed. **(A)** Within 2 minutes after the beginning of DAMGO application, average mEPSC frequency was 87.5 ± 6.2 % of the control section (CTRL 100 %; P>0.05; RM ANOVA on Ranks and Student-Newman-Keuls test). During the following 25 mins of wash-out, mEPSC frequency further dropped so that in 2 later intervals (wash 10 and wash 20) it reached a significantly lower value than when compared to the CTRL interval (CTRL 100 %; * P<0.05; RM ANOVA on Ranks and Student-Newman-Keuls test). **(B)** Pooling neighbouring intervals into longer sections did not show any significant changes in mEPSC frequency (P>0.05; RM ANOVA on Ranks and Student-Newman-Keuls test). **(C)** When compared to data from the control group, statistical testing showed that mEPC frequency within the DAMGO interval was significantly higher in controls (## P<0.01; RM ANOVA on Ranks and Student-Newman-Keuls test, paired t-test), in following intervals values of the two groups gradually evened out.

9.2.2. Incubation with CCL2 diminished the inhibitory effect of acute application of DAMGO.

Following the incubation with CCL2 (10 nM), the application of DAMGO (1 μM) caused only a small decrease in mEPSc frequency without statistical significance. The average mEPSC frequency recorded within the interval immediately following the application of DAMGO corresponded to 87.5 ± 6.2 % of the average mEPSC frequency in the control section of the recording (CTRL 100%; P>0,05; see Figure 9.6A). In the course of the wash-out period, mEPSC frequency fluctuated so that in two wash-out intervals the average frequency reached significantly lower values than in CTRL (CTRL 100%; P>0,05; see Figure 9.6A). However, there were no significant changes in mEPSC between neighbouring wash-out intervals. No significant change in mEPSC frequency was discovered even after pooling together neighbouring intervals of wash-out and DAMGO sections (P>0.05; see Figure 9.6B).

The response to DAMGO was also not homogenous amongst the neurons incubated with CCL2. From the total number of recorded cells (n=12), 9 reacted to the application of DAMGO by a decrease, and 3 by an increase in mEPSC frequency.

When compared to recordings made in neurons of the control group, the frequency of mEPSC (CTRL 100%) during DAMGO application is significantly higher in cells previously incubated with CCL2 (P<0,01; see Figure 9.6C). The differences were not significant between the data collected during subsequent wash-out intervals.

9.2.3. Acute application of CCL2 moderated the inhibitory effect of DAMGO.

When acute application of CCL2 (10 nM) preceded and paralleled the application of DAMGO (1 μM), a reduction in mEPSC frequency was still induced but lacked any statistical significance. The average values of mEPSC frequency measured within the DAMGO interval decreased to 75.7 ± 7.6 % of the precedent section of the recording (CCL2 100 %; P>0.05; see Figure 9.7A). From all 10 measured neurons, 9 reacted with the frequency decrease as described above, while the 1 remaining cell showed an increase in mEPSC frequency (124.7 % CCL2).

The average mEPSC frequency increased again during the following wash-out period and it reached 120.1 ± 24.5 % of CCL2 (P>0.05; see Figure 9.7A) by the 25. min of the wash-out. However, after pooling together longer intervals of the wash-out and DAMGO intervals, this increase is not as large due to great diversity amongst the measured cells (see Figure 9.7B).

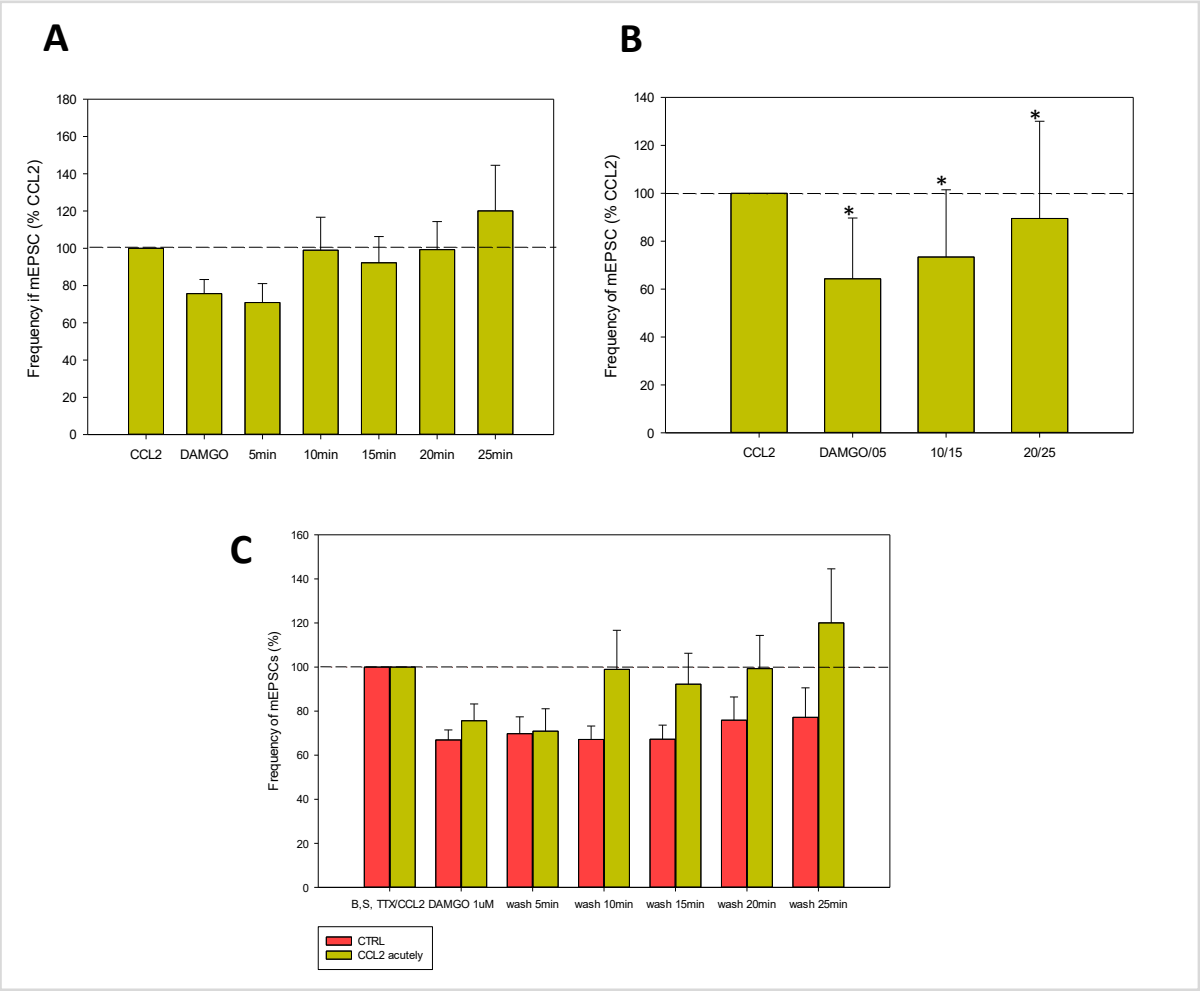

Figure 9.7: Acute application of CCL2 (10 nM) led to a decrease in mEPSC frequency following acute application of MOP-R agonist DAMGO (1 µM) but the inhibition lasted shorter than in the control group and the mEPSC frequency grew above the starting value in a subset of recorded cells**. (A)** Considering the values measured in the CCL2 interval as starting mEPSC frequency (CCL2 100 %), the decrease in activity after application of DAMGO is not significant, as well as differences between following intervals (n=10; P>0.05; RM ANOVA on Ranks). **(B)** After pooling the neighbouring intervals, the decrease after application of DAMGO compared to CCL2 values shows as significant for all wash-out intervals (n=10; * P<0,05; RM ANOVA on Ranks), but not between amongst the wash-out intervals. **(C)** When compared to data from neurons in the control group (with S,B,TTX interval setting the starting value, CTRL 100 %), simultaneous application of CCL2 (10 nM) and DAMGO (1 µM) led to a comparable decrease in average mEPSC frequency immediately after the application but showed faster recovery towards original values during the wash out phase. In the last wash-out interval (23.-25. min) the average mEPSC frequency surpassed the original values (120.1 ± 24.5 % of CCL2) but the difference between both measured groups was not great enough to prove any significance (P>0.05; t-test).

When compared to the development of mEPSC frequency recorded amongst neurons in the control group, the differences in mean values are clearly visible (see Figure 9.7C), especially between the 10. and the 25. min of the wash-out. However again, none of these differences were

statistically significant, probably because of the wide range of measured values amongst the acute CCL2 group. From the 10 neurons treated with acute application of CCL2, exactly a half (n=5) reacted with the increase above 100 % of CCL2 frequency, while the other half kept low mEPSC frequency similar to the data collected in the control group.

Surprisingly, the acute application of CCL2 didn't cause the immediate increase in average mEPSC frequency that was expected based on previous results published by our lab. The main issue is apparently the great variability of the recorded neuronal responses, which suggests that measured cells might belong to distinct neuronal populations. While 4 from 10 measured cells showed an increase in mEPSC frequency, in average up to 130.2 ± 3.6 % (CTRL 100 %; see Figure 9.7D), the rest reacted in the opposite way, with an average decrease in mEPSC frequency to 68.7 ± 8.8 % (CTRL 100 %; see Figure 9.7E). More neurons need to be recoded to explain these discrepancies.

9.2.4. Acute application of DAMGO had no effect on mEPSC amplitude, both in control neurons and after the treatment with CCL2.

In all the measured neurons, amplitudes slightly decreased with time during the wash-out phase, but the differences between neighbouring intervals were not statistically relevant. In control cells, the average amplitude in the first CTRL interval was 18.7 ± 1.5 pA, it dropped to 16.9 ± 1.3 pA in the DAMGO interval and by the last wash-out interval it was 15.1 ± 1.0 pA, which represents 86.9 ± 8.6 % of the initial value (CTRL 100 %; see Figure 9.8A). The neurons that were incubated with CCL2 showed similar development of the average amplitude, starting at 16.8 ± 1.3 pA during the CTRL interval, decreasing to 15.9 ± 1.2 pA after the application of DAMGO and further decreasing to 13.7 ± 0.7 pA by the end of wash-out (85.7 ± 6.4 % of CTRL; see Figure 9.8B).

The group of neurons treated with acute application of CCL2 showed very similar development of the average amplitude when compared to the average value measured in the CCL2 interval (CCL 100 %, see Figure 9.8C). There, the average amplitude was 16.3 ± 2.0 pA, the value dropped to 14.3 ± 0.6 pA after the application of DAMGO and by the end of the wash-out period ended at 13.6 ± 0.8 pA (80.0 ± 11.1 % CCL2). Interestingly, when the average amplitude was expressed in % of the initial amplitude measured during the CTRL interval, there was a noticeable increase in amplitude after the acute application of CCL2, up to 105.5 ± 13.1% (CTRL 100%; see Figure 9.8D). However, this change was not statistically significant.

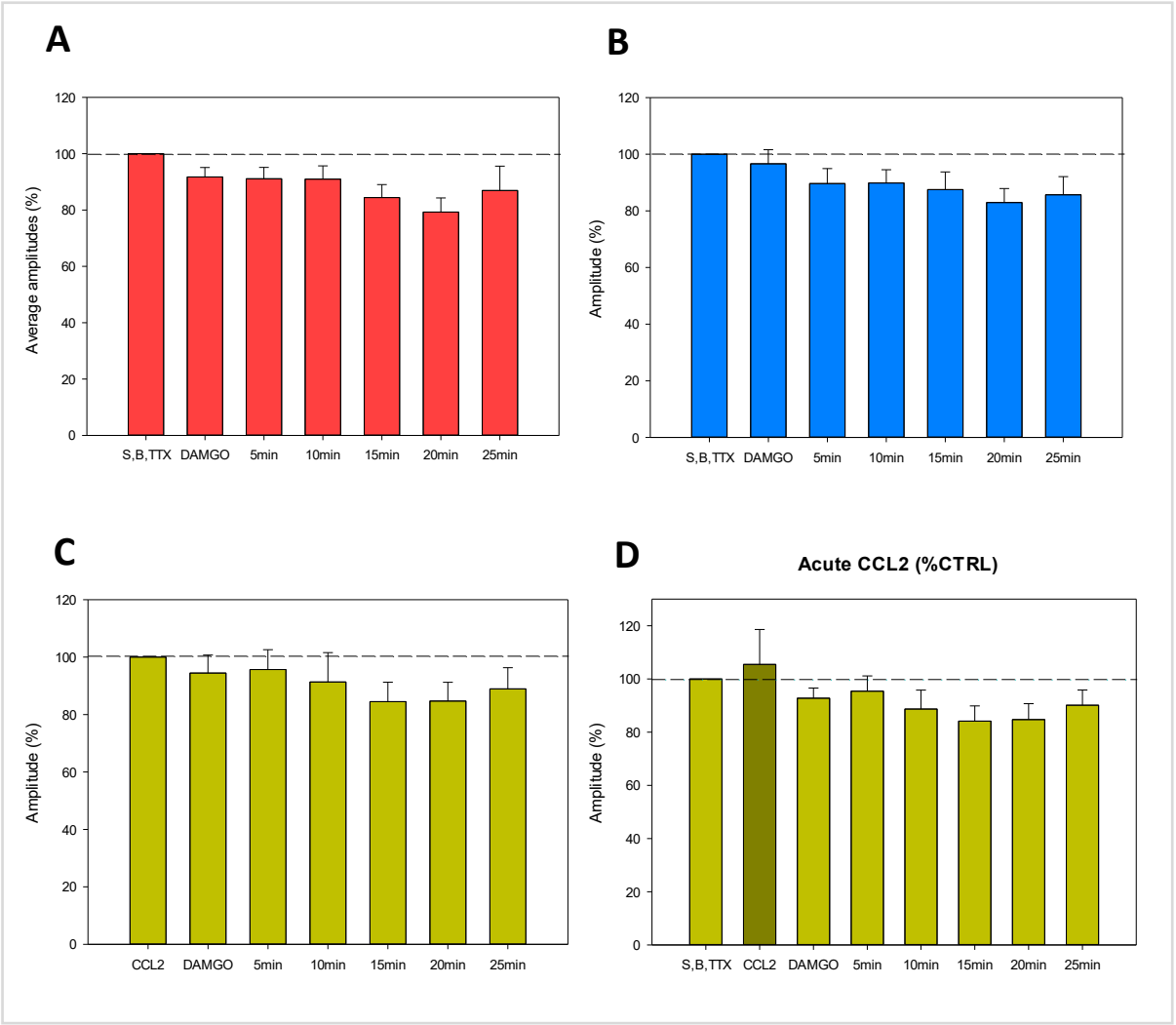

Figure 9.8: Application of MOP-R agonist DAMGO (1 µM) did not influence the amplitude of recorded mEPSC in secondary nociceptive neurons. **(A)** In the neurons of the control group, the average amplitude during the CTRL interval was 18.7 ± 1.5 pA and it did not significantly change after application of DAMGO or during the wash-out period (P>0.05; RM ANOVA on Ranks). By the end of the wash-out, the average amplitude was 86.9 ± 8.6 % CTRL. **(B)** In the neurons incubated with CCL2 (10 nM), the average amplitude during the CTRL interval was 16.8 ± 1.3 pA and it did not significantly change after application of DAMGO or during the wash-out period (P>0.05; RM ANOVA on Ranks). By the end of the wash-out, the average amplitude was 85.7 ± 6.4 % CTRL. **(C)** When referring to values measured during the CCL2 interval (CCL2 100%), the average amplitude of mEPSC in neurons treated with acute CCL2 application (10 nM) showed similar development, starting at 16.3 ± 2.0 pA and dropping to 80.0 ± 11.1 % CCL2 by the end of the wash-out. **(D)** When referring to values measured during the CTRL interval (CTRL 100%), we observed that the average amplitude increased up to 105.5 ± 13.1% of CTRL after the application of CCL2. However, this increase is not statistically significant (P>0.05; paired t-test).

9.3. Majority of the recorded neurons formed synapses with nociceptive fibers expressing TRPV1.

The majority of all measured neurons reacted to application of capsaicin solution (0.2 μM) in the end of the experiment by an increase in frequency of mEPSCs by 20% or more (a positive CAPS reaction). Since the increase of mEPSC frequency after CAPS application is mediated by TRPV1 channels located at central terminals of primary nociceptive neurons, CAPS sensitivity proves that recorded secondary neurons formed synaptic connections with TRPV1-expressing fibers.

Amongst the neurons isolated from animals with peripheral neuropathy, CAPS was applied to 11 control cells from which 10 showed a positive response, and to 9 cells previously incubated with CCL2 that all responded positively.

Amongst neurons isolated from the naïve animals, 12 control cells were treated with CAPS that all reacted positively. From 9 cells previously incubated with CCL2 that were treated with CAPS, 8 responded positively, and from 8 cells treated with acute CCL2 application that were treated with CAPS 6 showed a positive reaction.

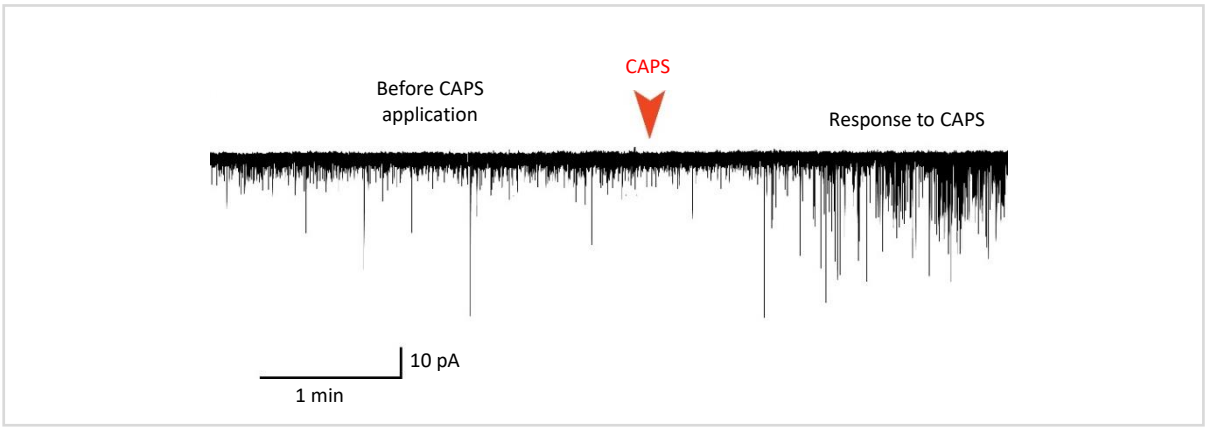

Figure 9.9: Majority of tested neurons reacted to CAPS application. A 5 min long excerpt from a single recording shows the time of CAPS application and the subsequent increase in neuronal activity. The temporary delay depends on the flow speed of the ECS and the exact localization of the recorded cell.

10. DISCUSSION

In the past two decades, research of nociceptive signalling payed a lot of attention to TRPV1 receptor, a cation channel that is mainly found in small diameter DRG neurons responsible for registration of painful stimuli. While the TRPV1 located in peripheral endings of DRG neurons had been extensively studied and its role in nociceptive signal transduction is well documented, much less is known about the TRPV1 expressed on central endings of these afferents. It has been reported that spinal TRPV1 participates on glutamatergic transmission at the first synapse formed by primary nociceptive fibers and secondary sensory neurons located in superficial laminae of the spinal dorsal horn (Baccei et al. 2003; Nakatsuka et al. 2002). Furthermore, increased activity of spinal TRPV1 had been closely linked to occurrence of various pathological pain states including pain caused by peripheral neuropathy. It is therefore assumed that TRPV1 sensitization and upregulation represents one of the mechanisms underlying modulation of synaptic activity at the first nociceptive synapse and thus contributes to development and maintenance of neuropathic pain (Kanai et al. 2005; Chen et al. 2009).

The activity of spinal TRPV1 can be modulated by a broad range of endogenous substances, some of them known to be upregulated in the spinal cord under pathological conditions (Spicarova and Palecek 2009; 2010). The research in our laboratory focuses on one of these substances, an inflammatory chemokine CCL2 that is known to participate on nociceptive processing following a peripheral nerve injury (Abbadie et al. 2009; Van Steenwinckel et al. 2011). The cooperation between TRPV1 receptors and CCL2 activated CCR2 receptors had been already demonstrated in cultures of DRG neurons and HEK293 cells (Jung et al. 2008; Kao et al. 2012). Using patch-clamp recordings from superficial dorsal horn neurons, our team had reported an increase in EPSC frequency following application of CCL2 that was prevented by pre-treatment with TRPV1 antagonist. These findings, supported by data from behavioural testing and immunohistochemical staining, indicate existence of functional cooperation between CCL2 and TRPV1 at the spinal cord level (Spicarova et al. 2014).

This work comprises of two blocks of experiments that aim to further describe the way CCL2 modulates spinal TRPV1 activity. The first part directly expands previous studies published by our laboratory (Spicarova and Palecek 2009; Spicarova et al. 2014) and concentrates on the CCL2 effect on TRPV1 activation by its endogenous agonist OLDA in animal model of peripheral neuropathy. The second part explores CCL2 influence on synaptic activity in the spinal cord following application of μ-opioid receptor agonist DAMGO. Activation of μ-opioid receptors (MOP-R) plays an important role in pain attenuation at the spinal cord level, and it has been recently

also implicated in modulation of TRPV1 activity (Bao et al. 2015; Endres-Becker et al. 2007; Vetter et al. 2008). Since the expression of TRPV1, MOP-R and CCL2/CCR2 overlaps in a population of nociceptive DRG neurons (Dansereau et al., 2008; Gao et al., 2009; Chen et al. 2006; 2008), we assume that DAMGO and CCL2 may interact in order to modulate the activity of spinal TRPV1.

10.1. Peripheral neuropathy leads to sensitization of presynaptic spinal TRPV1.

In the first block of experiments, we explored the effect of peripheral neuropathy on TRPV1 activation by its putative endogenous agonist N-Oleoyldopamine (OLDA) that is naturally found in central nervous system (Chu et al. 2003). We proceeded from a study previously published by our laboratory, which reported that an intrathecal injection of OLDA induces a pain-like behaviour in rats, which indicated that direct activation of spinal TRPV1 receptors by this agonist leads to changes in nociceptive synaptic transmission at the spinal cord level (Spicarova and Palecek 2009). Using patch-clamp recordings, the same study showed that under normal conditions, TRPV1 activation by OLDA is only possible when very high concentration of the agonist is used (10 μM), whereas considerably lower concentration of OLDA (0.2 μM) was sufficient to evoke a response after pre-treatment with an inflammatory mediator bradykinin or PKC activators (phorbol esters). In animal model of peripheral inflammation, low concentration of OLDA also proved as effective (Spicarova and Palecek 2009). Based on these findings, we assumed that spinal TRPV1 is sensitized under specific pathological conditions via PKC-mediated phosphorylation.

To confirm that similar TRPV1 sensitization occurs during peripheral neuropathy, we used patch-clamp recordings from superficial dorsal horn neurons in spinal cord slices isolated from animals with experimentally induced CCI. In our experiments we used recordings of mEPSC activity that is independent on generation of action potentials and does not involve activation of inner spinal circuits. TTX was used to block voltage gated sodium channels in spinal neurons and the effect of OLDA was measured as a change of mEPSC frequency in our experiments.

Following acute application of low concentration of OLDA, we observed a significant increase in average mEPSC frequency (120.6 ± 9.2 % of previous spontaneous mEPSC frequency) in neurons from animals with induced CCI neuropathy. The response to low concentration of the agonist suggested that TRPV1 receptors were sensitized, although the observed increase in mEPSC frequency is considerably less remarkable than the one previously documented in peripheral

inflammation (155.6 ± 5.4 %) and following a pre-treatment with bradykinin (177.5 ± 23.4 %) or phorbol ester (246.1 ± 40.6 %) (Spicarova and Palecek 2009).

When we tested the reactivity of the recorded neurons to CAPS, we observed an appropriate increase in activity in the vast majority of neurons, which indicates that we were indeed targeting neurons with inputs from TRPV1-expressing afferents. Nevertheless, it is possible that the response to OLDA was moderated due to changes induced by degeneration of some of the peripheral nerve fibers caused by the neuropathy and/or that the time after the CCI induction was not optimal. Another possible explanation for the weak response to OLDA is that the presynaptic TRPV1 receptors were not sufficiently sensitized under the conditions of peripheral neuropathy. It has been reported that experimentally induced CCI in rats mostly affects thicker Aβ and Aδ fibers, and less the small diameter nociceptive C fibers, although the extent of C fiber damage is very variable across individual animals (Obata et al. 2003). Since the reaction to OLDA was not homogeneous (only 10 neurons out of 12 reacted by increased activity), it is also likely that weaker average TRPV1 activation was caused by unequal TRPV1 sensitization amongst the measured neurons that were differently affected by the developing pathology. During the experiments we have targeted the lumbar segments of the spinal cord, where the effect of the CCI should be mostly expressed. It is possible that some of the neurons we have recorded were not directly affected by the neuropathy and recording higher number of neurons would probably enhance our understanding of this phenomena.

The age of experimental animals must be taken into account as well, since we were working with young animals (P24 – 28) that might react to CCI differently than adults and thus the level of TRPV1 sensitization might be less extensive. It was documented that rats about 3 weeks old are capable of much faster regeneration and adaptation following nerve damage than adult rats, although the maturity of their nociceptive pathways is already comparable (Fitzgerald 2005). Several studies even showed that rats younger than 3 weeks did not fully develop neuropathic pain, which suggests that some of the key mechanisms that underlie its development might yet not be functional (Fitzgerald 2005; Ririe and Eisenach 2006). On the other hand, it was documented that after 2nd week of age, rats with experimentally induced SNL manifest temporary mechanical allodynia associated with neuropathic changes indicating that at this ontogenetic stage the responsible mechanisms start to be effective (Ririe and Eisenach 2006). Furthermore, another study working with young rats (P20) clearly demonstrated significant lowering of nociceptive threshold already 5 days after the CCI surgery (Balasubramanyan et al. 2006). All facts considered, we assume that our experimental animals had already developed peripheral neuropathy, although TRPV1 sensitization might have been milder than it would have been in adults.

It was postulated that every miniature postsynaptic current represents a response to a single quantum of neurotransmitter released from the afferent, which means that mEPSC frequency corresponds to presynaptic activation. By contrast, the amplitude of a mEPSC represents mostly the strength of postsynaptic response of the recorded neuron (Edwards et al. 1990). Because we observed an increase in mEPSC frequency but average mEPSC amplitude showed no significant change following application of low concentration of OLDA, we assume that the agonist acted on presynaptic TRPV1 receptors. This is in agreement with data previously published in our laboratory (Spicarova and Palecek 2009).

Altogether, our results indicate that the effect of OLDA is mediated by presynaptic TRPV1 receptors that undergo sensitization under conditions of peripheral neuropathy, and it is quite likely that the sensitized spinal TRPV1 can be activated *in vivo* by naturally occurring TRPV1 agonists like OLDA. We further hypothesize that development of neuropathic pain might be to some extent dependent on TRPV1 activation and its modulation by local inflammatory mediators such as CCL2 that are elevated in the spinal cord as a result of peripheral nerve damage.

10.2. CCL2 modulates basal activity and TRPV1 response to OLDA in spinal neurons.

The second group of neurons isolated from neuropathic animals was incubated for at least 2 hours with CCL2 in order to test the effect of prolonged exposure to this inflammatory chemokine. In recordings from the control group, we confirmed that TRPV1 receptors were already sensitized by the neuropathic conditions, and it has been documented that acute application of CCL2 increased the activity of spinal TRPV1 (Gao et al. 2009; Spicarova et al. 2014). Therefore we predicted that additional incubation with CCL2 may lead to further sensitization of presynaptic TRPV1.

Firstly, we observed higher basal frequency of mEPSC and sEPSC in neurons incubated with CCL2 in comparison with neurons from the control group, although the difference was not statistically significant. This observation is in agreement with previously published studies showing that acute application of CCL2 is capable to induce an increase in mEPSC and sEPSC frequency in superficial dorsal horn neurons under normal conditions (Gao et al. 2009; Spicarova et al. 2014). Furthermore, we noticed that neurons incubated with CCL2 showed much greater decrease in EPSC frequency caused by the block of sodium channels with TTX, suggesting that higher CCL2 concentration somehow facilitates presynaptic generation of action potential. Since

this phenomenon is dependent on levels of intracellular calcium, we could hypothesize that presynaptic CCR2 activated by CCL2 stimulates activity of cation channels such as TRPV1.

However, we also observed a significantly bigger average amplitude of basal mEPSC and sEPSC in the cells incubated with CCL2 than in control cells. In their study, Gao et al. observed increased sEPSC amplitude after acute application of CCL2 that was nevertheless much less remarkable (Gao et al. 2009). Moreover, our observation is in contrast with data previously published by our laboratory that did not show any change in sEPSC or mEPSC amplitude following acute application of CCL2 in naïve rats (Spicarova et al. 2014). There are several possible explanations why in our case CCL2 treatment influenced both EPSC frequency and amplitude unlike in studies mentioned above. Since we were recording neurons isolated from neuropathic animals, the environment in the spinal cord contained elevated levels of pro-inflammatory mediators that could sensitize and/or upregulate CCR2 receptors expressed by spinal neurons (Gao et al. 2009; Zhu et al. 2014), which would subsequently lead to more intensive response to CCL2 through postsynaptic mechanisms. Or the number of CCR2 receptors in the postsynaptic membrane might be too small to produce any remarkable change in mEPSC amplitude after only brief application of CCL2, whereas longer incubation might be sufficient.

Following application of low concentration of TRPV1 agonist OLDA, we observed the expected increase in mEPSC frequency that was not accompanied by any significant change in average mEPSC amplitude, indicating once more the involvement of presynaptic TRPV1 receptors. Surprisingly, the final mEPSC frequency (132.8 ± 21.8 % of basal mEPSC frequency) was not significantly higher than the one observed amongst control neurons without the CCL2 treatment (120.6 ± 9.2 %). Furthermore, in spite of higher average value, the increase of mEPSC frequency did not reach statistical significance when compared to basal mEPSC frequency, which was caused by a considerably broad range of measured values. Probably more neurons would have to be recorded in order to gain clear evidence of the frequency change.

Because the onset of response to OLDA was variable amongst the incubated neurons, average mEPSC frequency was also calculated for intervals of maximum activity (160.5 ± 26.2 %) that already showed significant increase, although we still noted great variability in intensity of response to OLDA, which was remarkably more diverse than in controls. We hypothesize that this variability might be related to different levels of CCR2 expression in primary afferents. Since the dorsal horn neurons form variable number of synapses with afferents expressing at the same time TRPV1 and CCR2 receptors, it is quite likely that exposure to relatively highly concentrated CCL2 would lead to much higher increase of TRPV1-mediated activity in neurons with more TRPV1/CCR2-positive connections.

10.3. Activation of presynaptic μ-opioid receptor inhibits spinal nociceptive signalling.

In the second block of experiments, we focused on the joined effect of CCR2 activation by chemokine CCL2 and μ-opioid receptor (MOP-R) activation by its potent agonist (D-Ala2,N-Me-Phe4,Gly-ol 5)-enkephalin (DAMGO). Control recordings were made in superficial dorsal horn neurons isolated from naïve rats (P19-P23) with the intention to confirm the inhibitory effect of DAMGO application without the application of CCL2. We chose to use mEPSCs to evaluate the change in synaptic activity in order to avoid involvement of propriospinal neuronal circuits, and for MOP-R activation we applied 1 μM solution of DAMGO that has been previously reported as effective (Zhou et al. 2008).

As predicted, our data showed a significant decrease in mEPSC frequency (66.9 ± 4.6 % of CTRL) immediately following application of DAMGO. Because we didn't observe any change in amplitude in response to DAMGO application, we assume that its effect was mediated by presynaptic MOP-Rs that are known to represent over 70 % of all MOP-Rs found in the spinal dorsal horn (Dickenson 1995). All 13 recorded neurons homogeneously reacted to DAMGO by a robust decrease in mEPSC activity, which indicates that they formed connections with MOP-R-expressing afferents. Moreover, when we tested the reactivity of recorded dorsal horn neurons to CAPS, a positive reaction was detected in all cases, and thus we confirmed that the measured neurons formed synapses with TRPV1-expressing terminals. It is therefore likely that at least some of the afferents expressed both MOP-R and TRPV1, which would allow cooperation of both receptors.

The decrease in mEPSC activity that we documented in control neurons persisted during the whole wash-out phase (25 min), although we observed slow recovery of the mEPSC frequency towards the original values. Our findings are not in complete agreement with data published by Zhou et al., who have previously conducted a similar experiment on a comparable set of neurons (n=12; lamina II) and reported slightly stronger decrease in mEPSC frequency (51.5 ± 2.7 % of CTRL) with subsequent fast (approx. 8. minute of wash-out) recovery (Zhou et al. 2008). Furthermore, when repeating the experiment, the group documented an increase in mEPSC frequency above 150% of CTRL in 50 % of recorded lamina II neurons (n=18; Zhou et al. 2010). In addition, Zhou et al. also recorded mEPSCs in a group of neurons that were isolated from rats with RTX mediated degradation of TRPV1-expressing afferents and they reported that in these neurons the inhibitory effect of DAMGO persisted during the whole wash-out period (15-30 min) (Zhou et al. 2008; 2010). Their results were supported by a previous study showing that the effect of MOP-R agonists is stronger in isolectin B4 (IB4)-negative DRG neurons than in IB4-positive

ones that carry the majority of TRPV1 (Wu et al. 2004). Moreover, Chen and Pan showed that MOP-R agonists suppress mechanical nociception, which is generally attributed to IB4- afferents, more efficiently than thermal nociception mediated by TRPV1-positive afferents. They also documented that ablation of TRPV1-expressing DRG neurons by systemic application of TRPV1 agonist RTX reduced the number of MOP-Rs in presynaptic endings in the spinal dorsal horn but at the same time potentiated the analgesic effect of subsequent intrathecal injection of DAMGO (Chen and Pan 2006b). RTX treatment was also documented to prevent the development of morphine analgesic tolerance (Chen et al. 2007). Based on these data, Zhou et al. hypothesized that afferents that don't express TRPV1 show strong, persistent inhibition by DAMGO, but their reaction is masked in naive rats by the swift rebound increase in mEPSC frequency mediated by TRPV1-expressing afferents. They also suggested that imputs from TRPV1-expressing afferents play a key role in development of opioid tolerance.

Like Zhou et al., we also noticed a variability in the development of mEPSC frequency in the course of the wash-out, although the subset of neurons that showed an increase in mEPSC frequency above 100 % by the end of the wash-out was much less numerous (4 cells out of 13 measured) and most of the neurons showed a persistent decrease in mEPSC frequency. Since the experimental design we used was similar to the one of Zhou et al., it is likely that the difference in our data was caused by some procedural variability, for example if different subpopulation of spinal neurons were targeted. It is also possible that the discord is caused by different variety or age of experimental animals (Zhou et al. used Harlan rats within 7. – 8. postnatal week), different composition of used solutions, or different recording parameters.

10.4. CCL2 moderates the effect of DAMGO in spinal cord neurons.

To test the effect of chemokine CCL2 on the changes elicited by DAMGO in the superficial neurons of the spinal dorsal horn, we recorded mEPSC in two other groups of neurons: one group was previously incubated with CCL2, whereas the second group was treated with acute application of CCL2 preceding and paralleling application of DAMGO. We hypothesized that CCL2 would act via presynaptic CCR2 receptors and interfere with DAMGO mediated inhibition of nociceptive synaptic transmission.

We based our projections on previous studies that have reported the capability of many chemokines to induce heterologous desensitization of MOP-Rs in the periaqueductal gray (Heinisch et al. 2011; (Chen et al. 2007b), the dorsal horn of the spinal cord and in DRG (Rivat et

al. 2014). Heterologous desensitization describes an antagonistic relationship between two unrelated G protein-associated receptors expressed by one cell (such as CCR2 and MOP-R), which allows activation of one to cause inhibition of the other, usually through phosphorylation (Parsadaniantz et al. 2015). Specifically CCL2 has been already showed to moderate DAMGO-induced MOP-R activation in cultured DRG neurons (Zhang et al. 2004) and neuronal CCL2 in the spinal cord was implicated in the development of morphine tolerance (Zhao et al. 2012).

In the group of neurons from spinal cord slices that were previously incubated with CCL2, we still observed a decrease in mEPSC frequency following application of DAMGO (87.5 ± 6.2 % of CTRL), however the decrease was not significant. This was in contrast to the control experiments without CCL2 incubation, where significant, long-lasting depression of mEPSC activity was observed after DAMGO application. Moreover, when compared to the development of mEPSC frequency recorded from control neurons, the change in mEPSC activity within the DAMGO interval was significantly smaller than in the incubated neurons, indicating that the exposure to CCL2 interfered with MOP-R activation as we had expected. Similarly to the controls, the average mEPSC amplitude did not change in the course of the recording and the subsequent treatment with CAPS showed that majority of recorded neurons received inputs from TRPV1-expressing afferents. Therefore we would hypothesize that longer exposure to CCL2 leads to sufficient activation of CCR2 receptors located presynaptically on afferent terminals, which in turn stimulate signalling cascades of intracellular protein kinases. Kinases then phosphorylate MOP-Rs, leading to their internalization and/or TRPV1 receptors that become sensitized and induce stronger glutamate release from the presynaptic button.

The response to DAMGO was not homogeneous across the group incubated with CCL2 - from 12 recorded neurons, 9 reacted by a decrease and 3 by a slight increase in mEPSC frequency. We assume that the unresponsive cells might represent spinal neurons without synaptic connections to MOP-R-expressing afferents, or on the contrary neurons carrying high density of CCR2 receptors whose activation would reciprocally inhibit activity of present MOP-Rs.

Amongst the neurons treated with acute application of CCL2, the recordings showed an insignificant decrease in average mEPSC frequency following the application of DAMGO (75.7 ± 7.6 % of CCL2). Because 9 from the 10 tested neurons reacted to DAMGO application by a decrease of mEPSC frequency, we assume that most of tested neurons were contacted by MOP-R-expressing afferents. The documented change in synaptic activity was smaller than in controls but still bigger than in incubated neurons, although statistical testing didn't reveal any significant difference between these groups. Such mild response to CCL2/DAMGO might be explained for example by low representation of CCR2-expressing afferents contacting recorded neurons, or by

the fact that the brief exposure to CCL2 was not sufficient to cause more significant MOP-R inhibition.

Interestingly, by the end of the wash-out period we observed an increase in mEPSC frequency above 100 % in a subset of recorded neurons. We hypothesize that such hyperactivity might indicate a paradoxical excitatory effect of MOP-R agonists in the presence of CCL2, as it occurs during the development of neuropathic pain. However, due to a small number of recorded neurons and a too large variability of the responses, it was not possible to back up this assumption by sufficient data and more experiments are definitely needed.

In our experiments we did not reproduce the excitatory effect of acute application of CCL2. Our data showed only a mild decrease in mEPSC frequency during the CCL2 interval, which is in direct contradiction to earlier published studies clearly documenting an increase of EPSC frequency following acute CCL2 bath application (Gao et al. 2009; Spicarova et al. 2014). One of the possible explanations for this is erroneous targeting of deeper dorsal horn neurons that receive most of the input from myelinated afferent rarely expressing CCR2 (Jung et al. 2008; White et al. 2005). However, 6 neurons out of 8 that were treated with CAPS showed a positive response that indicated the presence of TRPV1-expressing afferents, and it is known that TRPV1 is almost uniquely found in thin unmyelinated or lightly myelinated fibers (Kobayashi et al. 2005). There is also a possibility that we targeted two distinct populations of superficial dorsal horn neurons, one with CCR2-positive contacts and one without, nevertheless when divided into CCL2 responsive and unresponsive groups, the number of measured neurons was not sufficient for a reliable statistical analysis. The population of CCL2 responsive neurons in our experiments did not overlap with the population of neurons showing hyperactivity by the end of the wash-out period.

11. CONCLUSION

Modulation of synaptic transmission in the superficial laminae of the spinal cord dorsal horn can substantially influence nociceptive signalling, especially under pathological conditions. Under these conditions activated neuronal and non-neuronal spinal cells release various substances that may trigger specific neuronal mechanisms leading to regulation of synaptic activity. While some of the key players of this modulatory system are already well-known, many are still to be explored and our results represent another small step in this process.

In this work, we present results of two distinct experiments that focused on the function of spinal TRPV1 receptor and its modulators. In the first part, we have used animal model of peripheral neuropathy to test activation of the presynaptic TRPV1 receptor by its endogenous agonist OLDA in low concentration (0.2 μM) that is ineffective under normal conditions. Patch-clamp recordings of mESPCs from superficial dorsal horn neurons isolated from animals with experimentally induced neuropathy showed that already such low concentration of OLDA evoked an increase in synaptic activity. This suggested that presynaptic TRPV1 receptors were sensitized under conditions of peripheral neuropathy. To test the possible influence of chemokine CCL2, we incubated the second group of neurons from neuropathic animals with chemokine CCL2. Under these conditions we observed increased basal EPSC frequency, as well as a stronger response to OLDA, which led us to the conclusion that CCL2 might further influence presynaptic TRPV1 sensitivity during peripheral neuropathy.

In the second part of the study, we tested the effect of selective μ-opioid receptor agonist DAMGO on synaptic transmission in the spinal cord dorsal horn. In control neurons we observed a persistent inhibitory effect of DAMGO on mEPSC frequency. This effect was diminished in neurons from slices previously incubated with CCL2, indicating possible interference with processes triggered by MOP-R activation. Acute CCL2 application had much weaker effect. Nevertheless, our results support the hypothesis that CCL2 and DAMGO might act antagonistically on the same target, possibly on the presynaptic TRPV1 receptor.

Our results suggest that TRPV1, CCL2 and MOP-R play an important role in modulation of nociceptive signalling on presynaptic endings of primary nociceptive afferents in the spinal cord dorsal horn. Also, their interaction at the spinal cord level may conduce to different pathological pain syndromes. Therefore, the results presented here contribute to our deeper understanding of these processes. Further research in the suggested direction might in future help discover new analgesic approaches for efficient treatment of chronic pain.

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