Charles University

Faculty of Science

Study Programme: Animal Physiology

MSc. Monica Pontearso

Modulation of nociceptive signalling on a spinal cord level under normal and pathological conditions

Modulace nociceptivní signalizace na míšní úrovni za normálních a patologických podmínek

DOCTORAL THESIS

Supervisor: Mgr. Diana Špicarová, Ph.D.

Institute of Physiology

Czech Academy of Sciences

Laboratory of Pain research

Prague 2024

Declaration:

I hereby declare that this PhD work was written by me and no other sources than quoted were used. Nor the thesis or any substantial part of it has been used to get any other academic title.

Prohlášení:

Prohlašuji, že tato disertační práce byla zpracována samostatně a nebyly použity žádné jiné než citované zdroje. Práce ani její podstatná část nebyla využita k získání jiného akademického titulu.

Prague, 21/04/2024

Monica Pontearso

Statement of co-authors

I certify that MSc. Monica Pontearso substantially contributed to the formation of the publications used as a basis of this thesis, and that her participation specified in the List of publications is correct.

21/04/2024 Mgr. Diana Špicarová, Ph.D.

Acknowledgements

I would like to state my sincere appreciation to my supervisor Diana Špicarová for her support during these 4 years and six months of PhD studies. She welcomed me starting from the first days in Prague, helping me finding an accommodation and solving all the administrative procedures needed in the Institute of Physiology. From a professional point of view, she gave me invaluable advices for planning experiments, understanding their outcomes, analysing data, preparing posters and writing papers and grant proposals. I would like to also thank my boss MUDr. Jiří Paleček, who followed me in this pathway giving me other precious advices about experiments, interpretation of data, my scientific carrier and how to evaluate a working place for my next job as postdoc. I am thankful for the opportunity to have learnt a technique such as patch clamp and many others and to have took part in interesting projects.

I appreciated also the collaboration with my colleagues Anirban Bhattacharya, Pavel Adámek, Jakub Slepička, Daniel Vasconcelos and Kateřina Kramerová.

I would like to dedicate a special appreciation space to Anirban Bhattacharya who guided me a lot into the world of the molecular biology, teaching me PCR methods but also how to become a more independent researcher and leave behind me the PhD student.

Special thanks to my family, who listened to all of my problems, supported me and tried their best to be next to me even though the long distance between Sicily and Prague.

Abstract

Present pain therapies are often insufficient and poorly managed, mainly for neuropathic pain treatment. In the past years, research has provided a body of evidence on the association of nerve injury and inflammation with mobilized immune reactions. Many cytokines and hormones are released during injury or inflammation, and these factors regulate neuronal encoding of painful sensations. For example, in pre-clinical models, inflammatory cytokines cause or amplify nociception by enhancing neuron excitability. Besides increased excitability via direct neuronal sensitization, it has also been demonstrated that a reduction of synaptic inhibition (disinhibition) occurs naturally in the course of neuropathic and inflammatory pain states. The spinal cord dorsal horn (SCDH) is the first relay station of the ascending pain pathway and a crucial modulatory site of painful stimuli transmission.

Three main aims characterize this doctoral thesis; each focused on the modulation of spinal nociceptive transmission by different molecules fundamental for pain processing. The **first** aim was to study the role of proinflammatory cytokine macrophage migration inhibitory factor (MIF) in nociceptive signalling following peripheral neuropathy induced by chronic constriction injury (CCI) of the sciatic nerve. The **second** aim was to investigate the role of anandamide (AEA) in the modulation of excitatory synaptic transmission at nociceptive synapses in the superficial spinal cord dorsal horn after carrageenan-induced peripheral inflammation. The **third** aim was to study the modulatory role of the cannabinoid receptor 1 (CB_1) regulatory protein, SGIP1, at first nociceptive synapses of the pain pathway in inflammatory conditions.

In vitro experiments were performed using whole-cell patch-clamp recordings from superficial dorsal horn neurons in acute spinal cord slices, supplemented by immunohistochemistry, PCR method, and in vivo behavioural tests to accomplish these goals.

Our results showed that a systemic administration of MIF inhibitor, ISO-1, attenuated mechanical and thermal hypersensitivity induced by CCI of the sciatic nerve in males but not in female mice. Moreover, ISO-1 administration partially restored the loss of balance between excitatory and inhibitory synaptic transmission in the superficial dorsal horn and decreased macrophage infiltration to the site of peripheral nerve injury and dorsal root ganglion (DRG). In addition, inhibition of MIF activity regulated signs of neuroinflammation. Concerning the second aim, our published data demonstrated that peripheral inflammation promoted the AEA-

mediated inhibitory effect in the spinal cord dorsal horn. Furthermore, SGIP1 protein deletion enhanced the inhibition of spinal nociceptive transmission induced by CB_1 receptor activation.

The results of this PhD study revealed that modulation of neuroinflammation and nociceptive synaptic transmission by MIF, AEA and SGIP1 protein plays an essential role in the nociceptive signalling after peripheral neuropathy and inflammation. These findings need further implementation to contribute to chronic pain treatments.

Abstrakt

Léčba bolesti je i v současné době často nedostatečná, zvláště pokud je vyvolávací příčinou neuropatie. V posledních letech se množí vědecké důkazy o spojení poranění nervů s neuroinflamací a mobilizací imunitních buněk. Během poranění nebo zánětu se uvolňuje mnoho cytokinů a hormonů, které regulují nociceptivní signalizaci vedoucí k vjemu bolesti. V preklinických modelech, prozánětlivé cytokiny vyvolávají nebo zesilují nocicepci zvýšením dráždivosti neuronů. Kromě zvýšené excitability neuronů procesem senzitizace, bylo také prokázáno, že v průběhu neuropatických a zánětlivých bolestivých stavů dochází ke snížení synaptické inhibice (disinhibice). V zadním rohu míšním jsou uloženy první nociceptivní synapse vzestupných drah bolesti, je to tedy významné modulační místo, kde je informace o přítomnosti bolestivého podnětu regulována.

Tuto disertační práci charakterizují tři hlavní cíle, každý je zaměřen na modulaci míšního synaptického přenosu, avšak různými molekulami, zásadními pro zpracování bolesti. **Prvním cílem** bylo studovat úlohu prozánětlivého cytokinu - inhibičního faktoru migrace makrofágů (MIF), v nociceptivní signalizaci po navození periferní neuropatie vyvolané chronickou konstrikcí sedacího nervu. **Druhým cílem** bylo studovat úlohu anandamidu (AEA) v modulaci excitačního synaptického přenosu na prvních nociceptivních synapsích v superficiální oblasti zadního rohu míšního po periferním zánětu navozeném aplikací karagenanu. **Třetím cílem** bylo studovat modulační úlohu regulačního proteinu kanabinoidního receptoru 1 (CB_1), SGIP1, na prvních nociceptivních synapsích rovněž u zánětlivých stavů.

K dosažení těchto cílů byly provedeny elektrofyziologické pokusy in vitro, kdy byly v akutních míšních řezech snímány postsynaptických proudy z celé buňky metodou terčíkového zámku. Tyto pokusy byly podpořeny biochemickými metodami (imunohistochemie, PCR) a doplněny in vivo behaviorálními testy.

Naše výsledky ukázaly, že systémové podání inhibitoru MIF, ISO-1, tlumilo mechanickou a tepelnou hypersensitivitu navozenou chronickou konstrikcí sedacího nervu u samců, ale ne u samic myší. Kromě toho aplikace ISO-1 částečně obnovila rovnováhu mezi excitačním a inhibičním synaptickým přenosem v superficiální oblasti zadního rohu míšního a snížila infiltraci makrofágů do místa poranění periferního nervu a spinálních ganglií. Inhibice aktivity MIF ovlivnila rovněž projevy neurogenního zánětu. Pokud jde o druhý cíl, naše již publikovaná data ukázala, že při periferním zánětu je inhibiční účinek aplikace AEA na nociceptivní

synaptický přenos zesílen. Kromě toho delece proteinu SGIP1 zesílila inhibici míšního nociceptivního přenosu indukovanou aktivací receptoru CB_1.

Výsledky této doktorské studie odhalily, že modulace neuroinflamace a nociceptivního synaptického přenosu prostřednictvím cytokinu MIF, AEA a SGIP1 proteinu hraje zásadní roli v nociceptivní signalizaci vyvolané periferní neuropatií nebo zánětem. Tato zjištění vyžadují další implementaci, aby mohla účinně přispět k praktické léčbě chronické bolesti.

LIST OF SYMBOLS AND ABBREVIATIONS

AEA	anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	Analysis of variance
2-AG	2-arachidonylglycerol
CGRP	calcitonin gene related peptide
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
CCI	Chronic Constriction Injury
CCL2	Chemokine (C-C motif) ligand 2
CD206	Cluster of differentiation 206
CD68	Cluster of differentiation 68
CD74	Cluster of differentiation 74
CNS	central nervous system
COX	cyclooxygenase enzymes
CSF	cerebrospinal fluid
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DRG	dorsal root ganglia
eCBs	endogenous cannabinoids
ECS	endocannabinoid system
EPSC	excitatory postsynaptic currents
FAAH	fatty acid amide hydrolase
GABA	gamma-amino-butyric acid
GPCRs	G protein–coupled receptors
GRK2/3	G protein-coupled receptor kinases 2/3
IASP	International Association for the Study of Pain
IL-1	interleukin-1
I.P.	Intraperitoneal

IPSC	inhibitory postsynaptic currents
IR	immunoreactive
ISO-1	(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester
le-IPSC	light-evoked inhibitory postsynaptic currents
mEPSC	miniature excitatory postsynaptic currents
MIF	macrophage migration inhibitory factor
NAPE	N-acyl phosphatidylethanolamine
NAPE-PLD	NAPE phospholipase D
NMDA	N-metyl-D-aspartate
NO	nitric oxide
PAG	periaqueductal gray
PIP2	phosphatidylinositol bisphosphate
PNI	peripheral nerve injury
PNS	peripheral nervous system
PWT	paw withdrawal threshold
sEPSC	spontaneous excitatory postsynaptic currents
SNI	spared nerve injury
Rag1	recombination activating gene 1
RVM	rostral ventromedial medulla
SCDH	spinal cord dorsal horn
SCI	spinal cord injury
SEM	Standard error of mean
SGIP1	Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting
TRPV1	transient receptor potential vanilloid type 1
TNF	tumor necrosis factor
TR	time of reaction
TTX	tetrodotoxin
VGAT	Vesicular GABA/glycine transporter
WDR	wide dynamic range

Contents

1 Introduction

Pain is a complex, physiological response to a threatening stimulus, which an endogenous or exogenous source could cause. After an injury, the body attempts to maintain its physiological properties involving regulatory mechanisms of the pain pathway. A pivotal role in the modulation of pain mechanisms is played by nociceptors, peripheral sensory neurons that transmit harmful stimuli from the periphery to the first relay station in the central nervous system (CNS), the spinal cord dorsal horn (SCDH).

Based on the extent of the stimulus, discrimination between acute and chronic pain is mandatory. If the unpleasant feeling lasts for more than three months and persists with the treatment, it is classified as chronic pain (Treede et al., 2019). In contrast to acute pain, which is a protective reaction, chronic pain is a maladaptive reaction and has no protective benefits. Neuropathic pain is a subtype of chronic pain and often results from an injury targeted to the peripheral or central nervous system. As a result, neurons release neuropeptides, chemokines able to activate and attract immune cells to the site of the injury, and viceversa, the immune cells modify neuronal activity. During this process, called neuroinflammation, glial cells have an essential part (Vergne-Salle & Bertin, 2021).

Nowadays, neuropathic pain is still poorly managed with current analgesics because of the complexity of this process. Moreover, most effective pain treatments, like opioids, are characterized by serious side effects and loss of efficacy (tolerance).

Even though knowledge of neuropathic and neuroinflammatory processes has notably increased in the last decades, the molecular nociceptive mechanisms are largely unknown. A better understanding of neuropathic pain mechanisms and neuroinflammation could lead to more effective and mechanism-based treatment approaches. Due to new discoveries about nervous and immune system interplay, focusing attention on cytokines mode of action and cytokines targeted treatments is important to develop new analgesic treatments.

For the above stated reasons, the aim of this Ph.D. project was to study underlying mechanisms of neuroinflammation and neuropathic pain development. The main focus was on the modulatory mechanisms of the spinal synaptic transmission in an established pain state, such as peripheral neuropathy, a phenomenon that attacks the nerves outside the brain or spinal cord.

The main investigation of this study is about the inhibition of macrophage migration inhibitory factor (MIF) activity by (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) in a neuropathic pain state at the SCDH, dorsal root ganglia (DRG) and sciatic nerve level in both sexes. We also focused our attention on the modulation of cannabinoid receptor 1 (CB_1), transient receptor potential vanilloid type 1 (TRPV1) channel and fatty acid amide hydrolase (FAAH) mediated by the endogenous lipid anandamide (AEA) in inflammatory conditions. The last part of this PhD work is an investigation of the CB_1 receptors regulation by Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting (SGIP1) protein in inflammatory conditions exploiting genetic deletion of SGIP1 protein. The experimental work was published in … original articles that are attached as an Appendix (page number).

1.1 The Pain Concept

Pain modulation conveys information concerning the incidence or threat of damage and, consequently, is one of the nervous system's essential functions. The current definition of pain by the International Association for the Study of Pain (IASP) is "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Every painful experience is arbitrary. Each person acquires the word's usage from early-life experiences involving injuries. It is, beyond any doubt, a sensation in one or more areas of the body, but it is also consistently unpleasant, making it also a psychological experience (Raja et al., 2020).

Classifications of pain most frequently used are the temporal and physiological ones (Wall et al., 2006). Acute pain plays a crucial role in preventing tissue damage and is the body's main line of defense against harmful stimuli. Failure to feel pain, primarily caused by genetic mutations, leads to a limited life expectancy and a life characterised by hospitalizations and medical therapies. In contrast to what was mentioned above, there are various pathological conditions, including those brought on by long-term inflammatory tissue damage or nerve injury following trauma, metabolic dysfunction, pathogenic infections, or cancer growth, in

which pain might persist and develop into a chronic condition. Chronic pain is a serious medical condition that afflicts people worldwide and is a significant cause of suffering (Kuner & Kuner, 2021). Briefly, acute pain is a physiological response of our body, while chronic pain is a pathological response of our body to a stimulus which is perceived as a threaten. Another difference applies to the length of the pain. Acute pain lasts from some minutes to less than six months, while chronic pain lasts for more than three months.

It is inevitable to distinguish between nociceptive, inflammatory, and neuropathic pain based on various features characterising the pain. Usually, pain occurs when signals from thinly myelinated (Aδ) and/or unmyelinated (C) nociceptive fibres reach the conscious brain. The feeling (pain) corresponds to the stimulus (noxious). The result is considered "normal" or "nociceptive" pain. A primary injury or disease in the somatosensory nervous system is the source of, or the cause of, neuropathic pain. Several mediators released around the area of tissue inflammation cause the nociceptive pathway to be activated and sensitised, resulting in inflammatory-related pain.

The majority of clinically painful situations comprise a combination of pain components, making it impossible to classify pain in a clear-cut manner. For instance, neuropathic and inflammatory components are typically present in chronic pain. Inflammation causes inflammatory nociceptive pain after tissue injury. Inflammatory mediators, though, may harm the neurons and contribute to the development of neuropathic pain. And vice versa, damage to the nerve system may result in an inflammatory response and, through neurogenic inflammation, contribute to inflammatory pain.

1.2 Pain Anatomy, basic insights

Pain shares many similarities with other sensory pathways. The common route of all the ascending sensory pathways is to convey information from the periphery (skin, muscles or viscera) to the higher brain areas in which the signals will be processed. The first relay station of the sensory routes is the spinal cord, specifically, the dorsal horns (DH) of the spinal cord. Most body tissues include free nerve endings, called nociceptors, on primary sensory neurons (DRG neurons) that respond solely to harmful or potentially damaging stimuli. These nociceptive signals are then transferred to the spinal cord by specific nerve fibers, C and Aδ fibers, of DRG neurons. DRG neurons are pseudounipolar neurons, characterised by neuron

bodies with a single axon bifurcating into two branches, one distal and one proximal to the spinal cord. In the spinal cord, the primary afferent nociceptors synapse with second-order, dorsal horn neurons. Then the message is relayed by these projection neurons to higher brain centers such as the brain stem reticular formation, thalamus, somatosensory cortex, and limbic system through ascending routes. Thalamus and cortex are the first two brain centers involved in pain perception processes. Where the ascending pathways terminate, the descending ones originate. At the brain level, incoming signals from noxious stimuli are modulated and then transmitted caudally. The rostral ventromedial medulla (RVM), the dorsolateral pontomesencephalic tegmentum, and the periaqueductal gray (PAG) area are supraspinal structures of the descending pain routes (Bourne et al., 2014). Ascending and descending pain pathways share the same route (from spinal cord to the brain and viceversa) but in opposite directions. In fact, descending pain pathways terminate at the SCDH level. The main function of the descending pain pathways might be to facilitate or inhibit the pain based on the neurotransmitters involved in the modulation of the signal (Bannister & Dickenson, 2016).

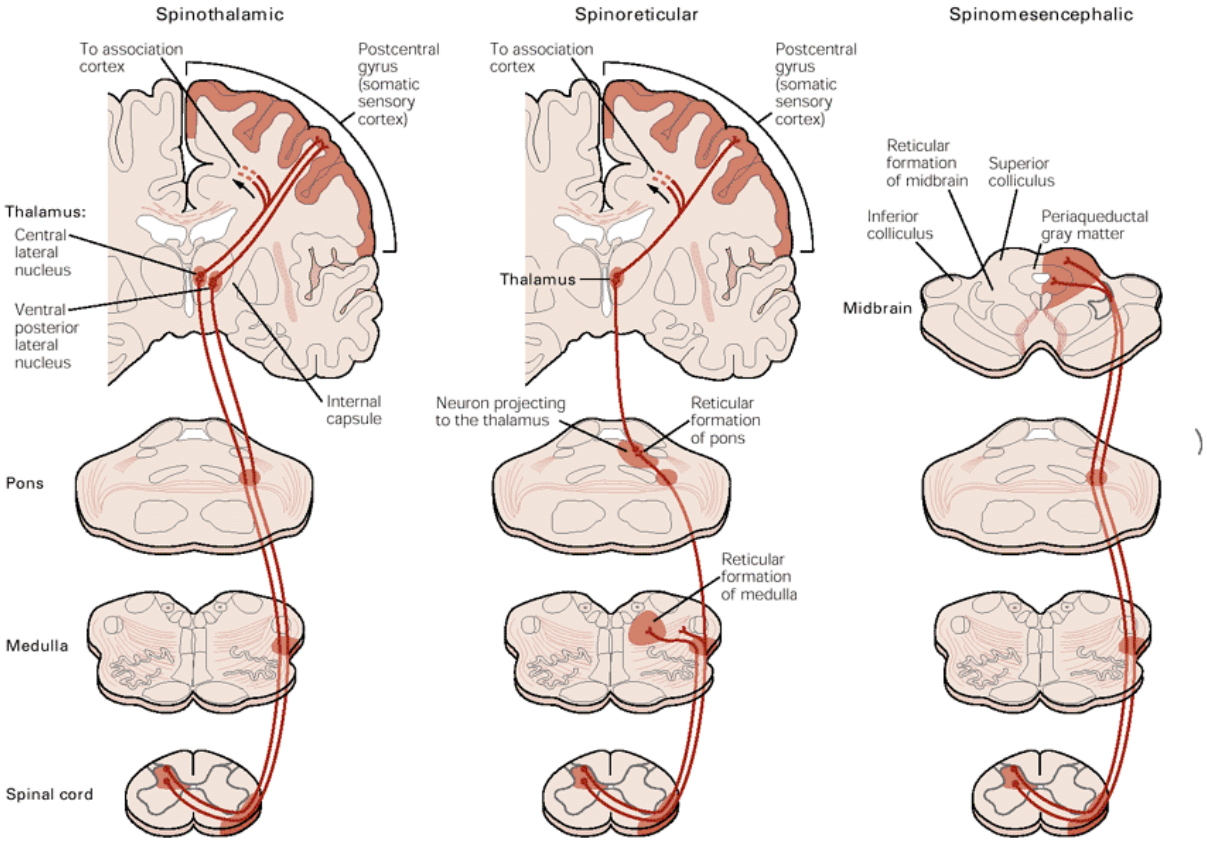

Fig. 1.1 Ascending pain pathways (Reprinted from Kandel, 2013)

2 Somatosensory Pain Pathways

2.1 Ascending and descending pain pathways

There are five pathways which allow the transport of the nociceptive information from the spinal cord to the higher brain areas, and these are: the spinothalamic, spinoreticular, spinomesencephalic, cervicothalamic, and spinohypothalamic tract. The main ascending nociceptive pathway is the spinothalamic tract and process sensory discriminative and affective motivational components of pain. It contains the axons of nociception-specific, thermosensitive, and wide dynamic range (WDR) neurons (Kandel, 2013). Two pathways carry nociceptive information to multiple thalamic nuclei that function as the main relay center of nociceptive information processing, the lateral and medial nuclear, and are part of the spinothalamic pathway (Millan, 1999). In the spinomesencephalic tract projection neurons create connections in the periaqueductal gray and the midbrain's reticular formation. It is a direct route that plays a significant role in the inhibitory modulation of nociception through the activation of descending inhibitory pathways (Willis & Westlund, 1997). Higher brain areas process and regulate nociceptive information exploiting descending pathways that project from the brain to the dorsal horn and either facilitate or inhibit synaptic communication. The periaqueductal gray of the midbrain is in charge of all descending routes (Steeds, 2016). Descending pathways are distinguished by the neurotransmitter they express in monoaminergic and non-monoaminergic pathways (Millan, 2002).

The conscious pain perception is processed in to the cortical regions of the brain, in which nociceptive information is conveyed. For pain perception, the cortical regions that play the main role are the primary and secondary somatosensory cortex, insular cortex, anterior cingulate cortex, and prefrontal cortex (Treede et al., 1999).

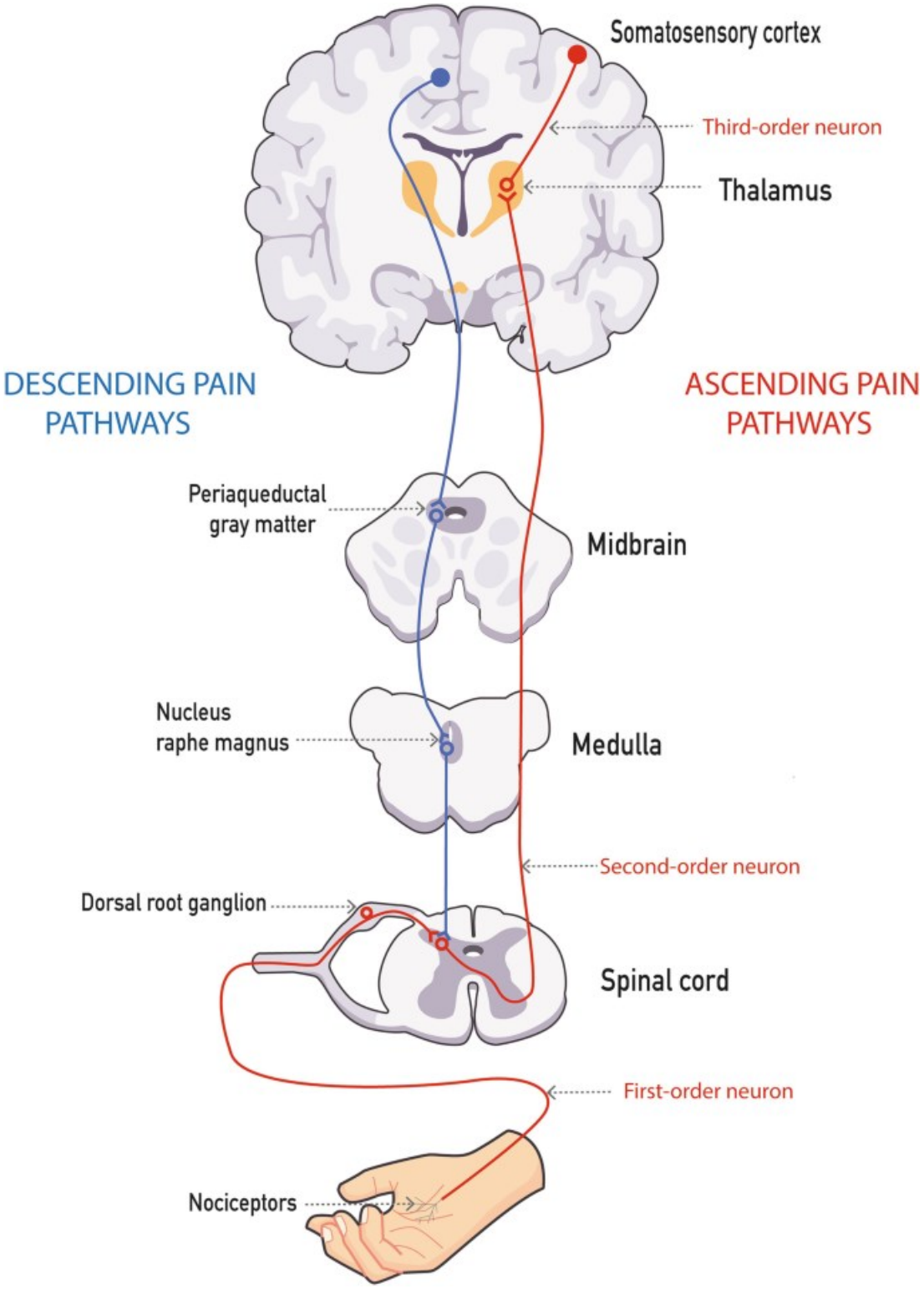

Fig. 2.1. Ascending and descending pain pathways (Reprinted from Torres-Méndez et al., 2022)

2.2 Nociceptors

Nociception is a neuronal process in which nociceptors acquire and transfer information about potentially harmful stimuli to the CNS. The term nociceptor was introduced by Sir Charles Sherrington in 1906 when he anticipated the existence of somatosensory neurons that are activated only in case of tissue damage or injury. In the early 1950s, Y. Zotterman and A. Iggo were the first to record extracellular electrical signals from living animal nociceptive nerve fibers, and all knowledge on mechanical and thermal sensitivity, as well as electrical features of these primary afferent fibers has come from analogous investigation (Kress & Reeh, 1996).

Loeser and Treede (Loeser & Treede, 2008) define a nociceptor as a sensory receptor able to transducing and encoding painful impulses. Nociceptors are specialized receptors localized at the terminals of the primary afferent fibers, which can be distinguished in C and Aδ fibers, of DRG neurons. C fibers are unmyelinated, small diameter and, as consequence, with a slow conductivity. Their signal transmission velocitiy ranges from 0.5 to 2.0 ms-1 and they are responsible for delayed, dull (blunt), and burning pain. Aδ fibers are myelinated, conduct impulses associated with sharp pain (pressure and cold) and have a rapid conductivity of 12-30ms-1 (Millan, 1999).

2.3 From Nociceptors to Spinal Cord

Nociceptors convert external inputs into electrochemical signals to transmit to second order neurons at the SCDH level. The nociceptive information travels along axons of the nociceptive DRG neurons, which are the first order neurons of the ascending pain pathway. Cell bodies within ganglia gather alongside adjacent glial cells, which supply essential metabolic support. Axons split into T-shaped branches, with both branches operating as a single axon. Individual axon branches are known as the distal and proximal processes. The distal process, or long peripheral process, spreads to the periphery, where the axon generates sensory endings in the innervated organ. The proximal process reaches the spinal cord. Nociceptive DRG neurons release excitatory neurotransmitters (glutamate) from their spinal terminals. These transmitters activate the second-order pain-transmission cells located mainly in the lamina I of the SCDH. The SCDH is divided into six laminae accordingly to their anatomical and electrophysiological

properties (Rexed, 1952). The most significant dorsal horn layers are the superficial laminae (laminae I and II), where nociceptive afferent fibers of Aδ and C types form connections with projection neurons. Lamina I, also known as the marginal zone, is composed mostly of large diameter projection neurons that receive input from Aδ and C primary afferent fibers. Based on identified distinctions in receptors expressed by spinal neurons and unique types of primary afferents, lamina II, or substantia gelatinosa, was further subdivided into lamina IIo (outer) and lamina IIi (inner). The majority of the neurons found in lamina II are spinal interneurons. Laminae III-IV are referred to as the proprius nucleus and receive inputs from the tactile or non nociceptive fibers, Aβ. Some neurons in the deep levels of the spinal cord, represented by laminae V-VI, are also engaged in nociceptive signalling (Millan, 1999).

2.4 Neuronal population in Spinal Cord Dorsal Horn

SCDH hosts various populations of neurons characterised by different morpho-functional properties.

Projection neurons are spinal cord dorsal horn neurons that transmit sensory information coming from afferent fibers to higher brain centers (Browne et al., 2021). They are categorized into three types based on the type of information they can receive and process. Aδ and C fibers forward a signal to high-threshold or nociceptive projection neurons, which are silent and can only be activated by noxious input transmitted by nociceptors. Nociceptive projection neurons are found mostly in laminae I and IIo, but to a lesser extent in laminae V and VI. Low-threshold or non-nociceptive neurons process sensory information from Aβ low-threshold mechanoreceptors. The largest number of low-threshold projection neurons are found in Laminae II-IV. Because of its ability to acquire and process not just direct input from Aβ and Aδ fibers, but also polysynaptic input from C-fibers, the last group of projection neuron is known as a WDR. WDR neurons in lamina V integrate peripheral and visceral nociceptive input. (Basbaum et al., 2009).

Another class of neuronal population in SCDH is represented by **interneurons** which further modulate the nociceptive transmission within the dorsal horn. Interneurons are generally classified as excitatory or inhibitory based on the neurotransmitters they secrete. Lamina II

contains the greatest number of interneurons in the spinal cord. Excitatory interneurons are principally responsible for the indirect polysynaptic stimulation of projection neurons. They secret excitatory amino acids as well as a range of neuropeptides. Inhibitory interneurons have direct contact with projection neurons and primary afferent fibers, allowing presynaptic and postsynaptic modes of suppression of nociceptive signaling from basic sensory neurons to be used. They are characterized by the neurotransmitters they produce, which are either gamma-amino-butyric acid (GABA) and glycine (Todd & Spike, 1993).

2.5 Anatomy of the Dorsal Root Ganglia

DRGs are allocated bilaterally to the spine and are primarily composed by the cell bodies of primary sensory neurons that are individually surrounded by satellite glial cells as well as by macrophages and endothelial cells. A thick connective capsule surrounds the entire structure. Morphological, electrophysiological, and neurochemical criteria were used to categorize DRG sensory neurons. DRG neurons can be divided into three types based on their somata size (Barabas et al., 2014): large-sized neurons (associated with Aβ and Aα fibers) with a large diameter (>50 μm); medium-sized neurons (associated with Aδ fibers and some C fibers) with a shorter diameter (30-50 μm) which are nociceptive neurons; and small-sized neurons (associated with C fibers) with a small diameter (10-30 μm).

2.6 Sciatic nerve functional structure

The sciatic nerve is made up of axons that are organized into nerve fascicles or nerve bundles. It has three protective connective sheath layers: epineurium, perineurium, and endoneurium (Fig. 2.1). The epineurium is the outermost layer of tissue that surrounds the entire peripheral nerve. Numerous nerve fascicles, blood arteries, lymphocytes, and fibroblasts are covered by the epineurium. The perineurium is the layer in the middle that extends around each nerve fascicle. Perineurium is a connective tissue with 7-8 concentric layers rather than a single layer. The innermost layer is the endoneurium which surrounds a group of tiny unmyelinated nerve fibers or myelinated nerve fibers (Q. Liu et al., 2018). The sciatic nerve is the biggest nerve in humans and rodents as well, arises from spinal nerves L4–S3, beginning in the lower back and radiating posteriorly through the legs until it reaches the heel of the foot (Giuffre et al., 2024).

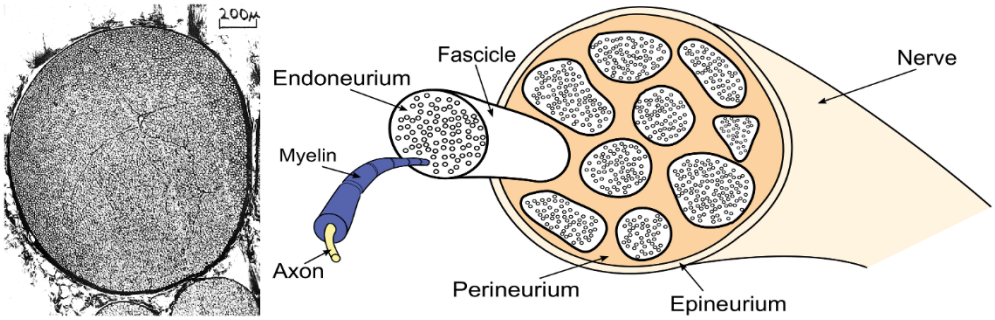

Fig. 2.2. On the left a cross section from Xenopus Laevis'sciatic nerve. On the right a schematic anatomical structure of sciatic nerves showing that the nerve stump is composed of epineurium, perineurium, and endoneurium (Reprinted from Troiani et al., 2018)

3 Pathological states

3.1 Neuroinflammation

Inflammation is a complex biological response to harmful stimuli such as injury, infection, and trauma. From a clinical point of view, inflammation is still considered as it was defined by Roman Celsus with five features: *calor, dolor, rubor, tumor and functio lesa*. These five cardinal signs are heat, pain, redness, swelling, and loss of function and after two millennia they are still used to describe the inflammation process (Mellor, 2012). The response of the body to the inflammatory stimulus is mediated by molecules of the innate and adaptive immunity such as cytokines, free radicals, eicosanoids, and transcription factors. Inflammation can be acute, protective, or chronic, unsafe for the body. When the harmful stimulus (injury, infection or trauma) occurs at the nervous system, inflammation is known as neuroinflammation.

Neuroinflammation is characterized by a variety of immune responses of the brain or spinal cord and involvement of specialized immune cells of the nervous system (Moyse et al., 2022). The cells involved in this process are resident glial cells in the central nervous system (microglia, oligodendrocytes, and astrocytes), non-glial resident myeloid cells (macrophages and dendritic cells), and peripheral leukocytes in the periphery. Neuroinflammation induces several events like microglia activation, synaptic alterations, neuronal death (Kaindl et al., 2008), nerve regeneration suppression, cognitive dysfunction disorders (Zhao et al., 2013),

increased Aβ production and Tau protein phosphorylation. Furthermore, neuroinflammation plays a role in CNS diseases and it is a common feature in most nervous system illnesses, particularly neurodegenerative diseases such as Alzheimer, Huntington, Parkinson, and amyotrophic lateral sclerosis (Zhang et al., 2023). In a neuroinflammatory state, one of the first mechanisms triggered in the brain as well as in the spinal cord is the modification in permeability of blood-brain-barrier which allows inflammatory cells to infiltrate into brain and spinal cord (Wu et al., 2021). The neuroinflammatory process starts with activation of local cells in the CNS like microglia and astrocytes and continues with invasion of neutrophils, lymphocytes, macrophages carrying out a secondary reaction to the inflammatory stimulus (Ransohoff et al., 2015).

Following peripheral nerve injury (PNI), immune reaction plays an important role at the spinal cord level. Once microglia cells are activated, they proliferate and change their morphology. The result is a secretion of pro and anti-inflammatory cytokines and interaction with spinal neurons. Previous studies have shown that there is a correlation between the intensity of the interaction and the location in the dorsal horns. In fact, the interplay between microglia and spinal dorsal horn neurons is strong in the superficial lamina of the dorsal horn (lamina I to III) and it becomes weaker in deeper dorsal horn laminae (Pottorf et al., 2022).

3.2 Nociceptive Pain

The first defense line against external threatening is conducted by nociceptors, sentinels that warn the body against some danger through a neural feedback that helps the central nervous system (CNS) to identify and prevent noxious and potentially harmful stimuli. These stimuli can be of different natures like chemical, thermal (heat or cold) or mechanical force and they have the common feature to activate the nociceptors.

Pain is classified into various categories based on the duration or on the origin of the stimulus: acute or chronic pain, nociceptive and neuropathic pain. While nociceptive pain concerns tissues damaged by physical or chemical agents such as trauma, surgery, or chemical burns, neuropathic pain is a consequence of diseases or damage targeting directly sensory nerves, such as shingles, diabetic neuropathy, or postherpetic neuralgia (Mitsi & Zachariou, 2016).

Nociceptive neurons communicate harmful information to the CNS in the form of high-threshold noxious impulses. The nociceptive signal may be routed to one of two distinct pathways: it may be given directly to a spinal reflex loop, resulting in a quick and reflexive withdrawal, or it may be delivered to parts of the brain responsible for integrating the information with higher-ordered perceptions such as pain (Tracey, 2017). Another possible outcome of painful stimuli is the release of chemical signals from nociceptive neurons' peripheral nerve endings (Frias & Merighi, 2016). Through the release of vesicles holding preformed pro-inflammatory cytokines and growth factors, this local activity will activate neighboring neuronal and non-neuronal cells (Woller et al., 2017a).

Specific unpleasant stimuli are sensed by expressed receptors that open their cation channels after the ligand-active site binding. Once activated, the channel depolarize the neurons, causing vesicle fusion and cytokine release. The released cytokines will stimulate secretion of other pro-inflammatory mediators from nearby endothelial, lymphoid, and epithelial cells (Frias & Merighi, 2016). Cells which receive the stimulus may then move or otherwise distribute pro-inflammatory signals, sensitizing or activating neighbouring nociceptors that were previously outside of the nociceptive area (Woller et al., 2017b; Armstrong & Herr, 2024).

3.3 Wallerian degeneration

Peripheral neuropathy occurs when a nerve in the body's extremities is injured. The damage can be caused by immunological or metabolic dysfunction (diabetes), traumatic nerve injury, drug-induced neuropathy (chemotherapy-induced peripheral neuropathy), HIV and post-herpetic neuralgia (Dubový, 2011). In the end, the common feature of all of these diseases will be neuropathic pain. Somatosensory nerve system injuries that affect its structure and function, causing pain to emerge spontaneously and reactions to noxious and harmless stimuli to be pathologically exacerbated trigger neuropathic pain. Pain indicates when the nociceptive system is facing maladaptive plasticity, which results in a variety of alterations that create a neurological disease state. Complex pain syndromes are characterized by multiple changes spreading throughout the neural system. Disinhibition and facilitation of synaptic transmission, ectopic action potentials production, neuroimmune interactions, development of new synapses and loss of synaptic connection and circuits are among the changes (Costigan, Scholz, et al., 2009). When the structure of a nerve fiber is interrupted, neuroinflammation begins and several

immune cells and pro-inflammatory molecules will be involved leading to nerve deterioration. Axonal injury generates a series of events, such as the proliferation of Schwann cells, the failure of the blood-nerve barrier, a reconfiguration of the endoneurial space as well as modifications in the endoneurial extracellular matrix, an increase in neurotrophin and cytokine production and the infiltration of circulating macrophages. Both molecular and cellular mechanisms distal to nerve injury create a milieu that promotes axonal regeneration (Dubový, 2011). Augustus Volney Waller investigated the characteristics of a severed frog nerve fibers, for the first time, almost 200 years ago. For this reason, this kind of injury is now known as Wallerian degeneration WD (Gaudet et al., 2011). Neuropathic pain and Wallerian degeneration share the same molecular and cellular alterations in the distal nerve endings.

When an axon is detached, the first responses lead to a process of self-destruction which is not dependent by immune cells surrounding the nerve. As a matter of fact, constriction of the nerve generates an impairment in axonal transport with axonal degeneration as a consequence. Axonal fragmentation within 2 days from the injury is caused by a rise in free intracellular calcium, calpains activation, and reduction of neurofilament and microtubular proteins (Coleman, 2005; Glass et al., 2002). Following the time course of the damage, Schwann cells start to disintegrate myelin sheath. Then, the next step is migration of macrophages to the distal stump, which will be finally cleared of degraded myelin (Stoll & Müller, 1999). In myelinated Schwann cells and peripheral nerve myelin axon growth inhibitors are present. For this reason, after peripheral nerve injury one of the most important mechanism is the clearance of myelin debris (Barrette et al., 2008).

As a consequence of Wallerian degeneration, not only in the distal nerve stump but also in DRG and spinal cord there is infiltration and activation of immune cells. At the nerve site immune cells such as mast cells, neutrophils and lymphocytes attract molecules like cytokines, chemokines and proteases which contribute to the initiation of inflammatory reaction, recruitment of neutrophils and monocytes/macrophages and development of hyperalgesia (Dubový, 2011).

Molecular, morphological and gene expression modifications characterize PNI at the spinal cord. After an injury of the nervous system, spinal microglia are quickly activated and, as a consequence, they upregulate synthesis and expression of several proinflammatiory cytokines like tumour necrosis factor α and interleukin 1β as well as cell surface receptors (Clark et al., 2007). Activation of cell-surface receptors on microglia surface in response to extracellular

ligands binding, intracellular signalling cascades are triggered. It was demonstrated that, after PNI, p38 is activated and has a role in neuropathic pain development (S.-X. Jin et al., 2003). To prove its role in neuropathic pain, injections or infusions of p38 inhibitors have been used to attenuate mechanical allodynia induced by PNI (S.-X. Jin et al., 2003).

3.4 Gate Theory of Pain Modulation and Disinhibition

After years of study on nociception and pain transmission, the gate control theory was published in 1965 by Melzack and Wall (Melzack & Wall, 1965). Because inhibitory interneurons form direct and indirect synaptic connections with projection neurons, their stimulation reduces outputs for higher control and reduces nociceptive transmission and pain. In this theory the gate represents the obstacle of nociceptive signal's travelling from the spinal cord to the higher brain centers. When inhibitory interneurons are blocked by nociceptive afferent inputs, the gate opens, allowing nociceptive signals to be transmitted (Braz et al., 2014; Melzack & Wall, 1965). Nonnociceptive fibers trigger an inhibitory interneuron in the DH, which closes the gate, which indicates the inhibition of nociceptive impulses from the spinal cord to the brain (and supraspinal nuclei) by nonnociceptive inputs and descending supraspinal control.

Disinhibition, in turn, suggests loss in GABAergic or glycinergic control because of inhibitory interneuron malfunction (Taylor, 2009). The result of reduced activity of these interneurons is an increase in pain perception. Several studies have revealed that disinhibition is one of the most important underlying mechanisms of neuropathic pain, including pain relief signals from GABA administration and pain-like behaviors in animal models when GABAA or glycinergic receptor antagonists are delivered intrathecally (Hwang & Yaksh, 1997). There are several reasons why disinhibition occurs, such as decreased afferent stimulation to inhibitory interneurons (Basbaum et al., 2009), death of inhibitory interneurons (Sandkühler, 2009), reduction of GABA in the synaptic cleft, specific glycine modifications and modified GABA function when released (Todd, 2010). In the spinal cord dorsal horn, loss of inhibition, also called disinhibition contributes to many pathological pain states which share peripheral hypersensitivity as common feature (K. A. Moore et al., 2002).

4 Animal Models of Pain

4.1 Neuroinflammatory Pain Model

Neuroinflammation can be either transient or persistent, based on the nature and extent of the stimulus and on the inflammatory cascade caused by this one.

Carrageenan model, one of the most used inflammation models, is characterized by the use of carrageenan as irritant molecule to be injected subcutaneous in the hindpaw of the murine organism. One feature is the local inflammation with five cardinal signs: swelling, hypersensitivity, redness, heat, and loss of function (Fehrenbacher et al., 2012)

To evaluate inflammation in an animal model, provide methods to identify an inflammatory state and to supply medical treatments to prevent these responses (anti-inflammatory drugs), some signs like swelling (edema) or hyperalgesia (hypersensitivity to a noxious stimulus) are used (Fehrenbacher et al., 2012).

Acute inflammation is caused by the overproduction of free radicals, the activation of complex enzymes, and the secretion of various inflammatory and pro-inflammatory mediators. A biphasic edema is usually generated by injecting carrageenan into the subplantar surface of a rat paw. The early phase (around 1 h) is associated with the release of serotonin, histamine, bradykinin, and, to a lesser degree, prostaglandins produced by cyclooxygenase enzymes (COX), while the delayed phase (after 1 h) is associated with continuation of prostaglandin production and the neutrophil infiltration (Gilligan et al., 1994). The delayed phase of carrageenan-induced acute inflammation is likewise characterized by the release of nitric oxide (NO), neutrophil-derived free radicals, and pro-inflammatory cytokines that include tumor necrosis factor (TNF-) and interleukin-1 (IL-1) (Halici et al., 2007). Multiple studies indicate that medications targeting free radical generation, the COX enzyme, and pro-inflammatory protein expression (e.g., inducible nitric oxide synthase) may give superior control over inflammatory conditions than currently available therapeutic treatments (Ronchetti et al., 2009).

4.2 Neuropathic Pain model

Neuropathic pain arises after a nerve damage caused by various stimuli like mechanical, chemical and thermal. In this chapter, there is a brief description of a model derived by a mechanical insult which is known as Chronic Constriction Injury (CCI) of the sciatic nerve. The CCI model has faced changes in the last 35 years since when Bennett and Xie (Bennett & Xie, 1988) developed this injury in laboratory animals for the first time. The first CCI model, the ligatures around the sciatic nerve were too tight and, as a consequence, lead to nerve strangulation and, epineurial oedema and consecutive peripheral nerve compression and reduction of epineurial blood flow. Bennett and Xie model was characterized by four chromic gut (4-0) ligatures tied around the sciatic nerve, and it is ineffective for identifying neuroinflammatory reactions caused by thread material (Clatworthy et al., 1995) and/or Wallerian degeneration (degeneration of the axon after the nerve lesion) of injured axons (Klusáková & Dubový, 2009). On the other hand, CCI of the sciatic nerve under aseptic conditions using 3-0 sterilized thread is a more suitable and reliable model for investigating neuropathic pain generated by traumatic nerve injury and related neuroinflammatory reaction as a result of Wallerian degeneration (Dubový, 2011).

After sciatic nerve injury, fibers loss is also a feature to consider and it involves in big part heavily myelinated Aα and Aβ fibres, and a smaller amount of Aδ and C fibres (Basbaum et al., 1991). In the corresponding DRGs (linked with sciatic nerve, L4-L6) afferent neurons with injured and uninjured axons are present. There is a mutual collaboration between injured and uninjured fibers at the nerve segment distal to the ligature because molecules released during the Wallerian degeneration are taken up by the intact afferent axons and retrogradely transported to the DRG neurons (Klusáková & Dubový, 2009).

5 Immune Cells

The immune system defends the body against bacteria and other substances in tissues, on skin and in physiological fluids. The immune system is made up of two activities which are different but work closely together: innate (generic) immunity and adaptive (specific) immunity.

5.1 Innate immune cells

Mast cells play an important role in the early phases of inflammation and the development of pain hypersensitivity associated with peripheral nerve injury. Mast cells are commonly thought to be crucial modulator cells in allergy illnesses, but they are also significant triggers and effectors of innate immunity and are found in a variety of tissues, including peripheral nerves (Galli et al., 2005). It has been shown that mast cells degranulate near the site of a nerve injury, secreting mediators such as histamine, serotonin, proteases, prostaglandins, and cytokines (Galli et al., 2005). Some mediators may stimulate nociceptors, leading to higher firing rates, whereas serotonin can cause hyperalgesia by directly activating nociceptors (Sommer, 2004). Several mast cell-derived substances are also chemoattractant, with the ability to attracting neutrophils, which then contribute to the production of more inflammatory mediators (Biedermann et al., 2000).

Neutrophils, known also as polymorphonuclear leukocytes, are abundant near the site of nerve injury and take part in the earliest phases of peripheral nerve injury, reaching its highest at 24 h (Zuo et al., 2003).

Reduction of circulating neutrophils at the time of peripheral nerve damage reduced the onset of neuropathic hyperalgesia considerably (Perkins & Tracey, 2000). Co-culture of dissociated DRGs and resting neutrophils results in a fast (within 10 minutes) shift of neutrophils to an active state triggering the inner oxidative burst, along with increased firing frequency and excitability of the neurons, leading to a rise in the number of apoptotic or injured neurons within 24 hours (Shaw et al., 2008). The generation of reactive oxygen species by neutrophils contributes to the inflammatory process (C. Nathan, 2006). TNF, IL-1, IL-2, and IL-6 are cytokines released by activated neutrophils that can directly stimulate neurons and cause cellular damage (Murata et al., 2006).

In the immune system, **macrophages** carry out a number of functions, including antigen presentation, phagocytosis, and cytokine generation (Ji et al., 2016). Macrophages size is variable and it depends on the condition of the tissue (healthy or inflamed): their diameter varies from 14 to 50 um (Leskovar et al., 2001). Macrophages are formed by monocyte differentiation and phagocytize particles such as dead or dying fragments of axotomised axons and damaged Schwann cells (Brück, 1997). Furthermore, it was demonstrated that macrophages in injury-related processes act later than mast cells and neutrophils, playing a role in the fundamental

events of phagocytosis and Wallerian degeneration, both of which are required for peripheral nerve regeneration. Upon nerve injury, infiltration of hematogenous macrophages assists the local macrophages (which do not multiply significantly) in removing cellular debris (Bendszus & Stoll, 2003). In fact, blood-derived macrophages penetrate the DRGs at later time points, with their numbers increasing threefold one week after CCI in rats (Hu et al., 2007). The migration phase starts slowly, usually within 24 hours, proceeds for up to 3 days, and propagates distal from the injury site (Zuo et al., 2003). Macrophages are involved in pain-related behavior in addition to phagocytosis and nerve regeneration. For example, pharmaceutical macrophage depletion or genetically impaired macrophage response resulted in decreased pain hypersensitivity within various neuropathy models (Barclay et al., 2007; T. Liu et al., 2000). TNF, IL-1, and IL-6 are only a few of the cytokines secreted by macrophages that have been linked to hyperalgesia (Sommer & Kress, 2004). It has been shown that perineural injection of nicotine (a macrophage activation inhibitor) reduced augmented production of IL-1 in the sciatic nerve and also the establishment of pain hypersensitivity in PNL-injured mice (Kiguchi et al., 2010). Reactive oxygen species and prostaglandins, which sensitize primary afferents, are implicated in pain and considered macrophage derived mediators as well (C. F. Nathan, 1987). Based on the stimulus and the type of cytokines released in the microenvironment, macrophages can switch phenotypes from pro-inflammatory M1-like to anti-inflammatory M2-like phenotypes (or viceversa), which perform different tasks in the management and development of pain.

5.2 Adaptive immune cells

The adaptive immune response is composed by two main cellular elements, T and B lymphocytes. **T lymphocytes** carry out an essential role in cell-mediated immunity and B lymphocytes are principally involved in the synthesis of antibodies. T lymphocytes are categorized into different subpopulations: regulatory T cells (Tregs), cytotoxic T (Tc) cells helper T (Th) cells. In the last decades, involvement of T cells in several models of neuropathic pain has been shown (Jiang et al., 2022). Moreover, also the timing of T lymphocyte migration into injury site (*sciatic nerve*) was investigated, showing that the infiltration occurs by 3 days and reaches the *peak at day 21* (Moalem et al., 2004). Moalem et al. (2004) revealed, in a model

of CCI, that athymic nude rats lacking functional T cells experience considerably less mechanical allodynia and thermal hyperalgesia than heterozygous littermates. These findings have now been validated in both nude and recombination activating gene 1 (Rag1) T cell-deficient mice, which showed a considerable decrease in mechanical hyperalgesia after established neuropathic pain models (Costigan, Moss, et al., 2009). Other targets of T cells infiltration are also DRGs and spinal cord, as was reported after sciatic nerve transection or ligation (Sweitzer et al., 2002). In the *spinal cord*, the peak of T cells invasion was achieved after *7 days* (Cao & DeLeo, 2008), which is considered a presumably significant time-point in the pain chronicity progression (Austin & Moalem-Taylor, 2010). The infiltration of T cells, following nerve injury, is essential for the initiation of mechanical sensitivity, as evidenced by a decrease in pain behaviors in T-cell defective mice, Rag1 knockout mice (Costigan, Moss, et al., 2009).

6 Cells involved in the immune response

Over the last decade, there has been a sudden increasing of studies that have given attractive evidence that neuropathic pain pathogenesis involves interactions between neurons,immune-like glial cells and inflammatory immune cells, as well as an extensive number of immune cell-derived inflammatory chemokines and cytokines. In fact, injury of peripheral nerve causes an immune response that has been recorded in a variety of anatomical areas, including the supraspinal sites associated with pain pathways, the spinal cord, DRG and the injured nerve (Austin & Moalem-Taylor, 2010).

6.1 Glial Cells

The nervous system is composed mainly by two classes of cells: glia (almost 90%) and neurones (almost 10%) (Butt & Bay, 2011). For almost two decades, glial cells were considered as inactive components of the CNS when their function were compared with the ones of the neurons. Researchers have been considering glial cells inactive or silent because they are not able to receiving and forwarding electrical signals but they are specialized in playing mostly supporting and nutritive duties. The development of advanced physiological tools, which

include fluorescent calcium dyes and the patch-clamp, has radically altered the perception of glia as 'inactive' cells of the brain.

Based on their origin, from neuronal (i.e. ectodermal) or non-neuronal (i.e. mesodermal) tissues, glial cells are divided into macroglial cells or neuroglial cells and microglia. Macroglial cells or neuroglial cells are located in the CNS and they include subgroups like astrocytes, oligodendrocytes and ependymal cells. The ventricles' walls in the brain and the central canal in the spinal cord are formed by ependymal cells. Ependymal cells are responsible for the creation and transport of cerebrospinal fluid (CSF), the formation of a dividing membrane between the CSF and CNS, and the passage of chemicals between the two compartments (Butt & Bay, 2011). Astrocytes bridge the gaps between neurons and maintain their structural stability. They are composed by structures, short processes, that connect to capillary blood vessels and are referred to as perivascular end feet. As a result of their proximity to blood vessels, they are assumed to be in charge of metabolite exchange between neurons and vasculature. Astrocyte function is important in modulation of pathological pain states. They can influence signaling pathways of glial cells, changing expression of transcription factors for example. Another role of the astrocytes is at the cell surface level, influencing expression of channel proteins and receptors. In the end, they modulate also the synthesis and secretion of cytokines and chemokines (Cheng et al., 2022).

Oligodendrocytes are in charge of producing myelin sheaths, a protective layer that enwraps around the axon (Michalski & Kothary, 2015).

Microglia are derived from macrophages, which infiltrate into the brain throughout development and settle in the CNS. According to dangerous stimuli or pathological conditions of the CNS, microglia are always recruited first than other glial cells (Colonna & Butovsky, 2017). When activated, microglia may secrete a wide range of oxidants, pro-inflammatory molecules, and possibly neurotoxic substances that contribute in the acute inflammatory process (Isik et al., 2023). Furthermore, microglial activation is important in subsequent CNS damage after injury in neuroinflammatory conditions during chronic states(Qin et al., 2023). Moreover, during chronic neuroinflammation, microglia can remain activated for an extended period of time, triggering neurodegenerative alterations via cytokines and neurotoxic chemicals release (Muzio et al., 2021).

Based on the part of the nervous system in which they reside, glial cells can be divided in other subgroups. The CNS is composed by macroglia and microglia, meanwhile the peripheral

nervous system (PNS) is composed by Schwann and satellite cells. Schwann cells, which enwrap and myelinate peripheral axons, are the most common type of glia in the PNS). Schwann cells have the same function of the oligodendrocytes, which is the production of the myelin sheath, but instead of doing it in the CNS, they do it in the PNS. They also take part in the phagocytosis of debris, which contributes to cleaning of the surrounding area. Neurons of the sensory system (DRG) and the autonomic system are surrounded by satellite cells, which regulate the chemical equilibrium in the area around those neurons (Hanani & Spray, 2020). Satellite glial cells have a unique feature in the body because they wrap around cell bodies of the neurons and sometimes they cover all the neurons' surface. They are present only around peripheral ganglia and the gap between satellite cells and neurons' surface is similar to the one of the synaptic cleft. This unique structure is called 'neuron–glial unit' and allows a mutual interaction between the neuron and the satellite glial cell (Hanani & Spray, 2020).

6.2 Sex differences in neuro-immune modulation of pain

It is now well known that there is a cross-talk between nervous and immune system, so that immune cells influence the neurons' activity and vice versa. Numerous studies have highlighted sex differences in immune cells activation during progression and maintenance of chronic pain (Rosen et al., 2017; Sorge & Totsch, 2017). In the past twenty years, investigations on chronic pain considering both sexes have been increasing. Researchers have focused their attention on gonadal hormones, concluding that estrogens are immunostimulatory while testosterone is immunosuppressive (Roved et al., 2017; Moulton, 2018).

Recently literature validated the importance of spinal T-cells in neuropathic pain model, but only in female mice, with male mice relying on microglial-mediated modulation of pain (Ji et al., 2016). Sorge tested paw withdrawal threshold (PWT) in mice of both sexes seven days after induction of neuropathic pain using spared nerve injury (SNI) as model. Moreover, they decided to inject intrathecally glial inhibitors seven days after the nerve injury and tested again PWT. The results showed a significant prevention of allodynia in male mice but not in female mice. Sorge wanted to test PWT also after induction of a persistent inflammatory model and minocycline systemically application. Mechanical allodynia was reversed again only in male but not female mice. To further prove involvement of spinal cord microglia in modulating allodynia in male subjects only, a transient depletion of microglia was made by intrathecal

injections of saporin toxin conjugated to macrophage antigen complex-1. The behavioural results were comparable to the ones obtained with glial inhibitors injections, so no reversal of allodynia in female but complete recover in male mice (Sorge et al., 2015). Other studies showed that spinal glial inhibition (using minocycline) reversed pain-like behaviour only in male mice (Fernandez-Zafra et al., 2019).

It is a direct consequence to think about an alternate neuro-immune pathway regulating the modulation of pain in female mice since several investigations have shown that every treatment aimed at decreasing microglia signaling prevented allodynia in male mice but not in female mice, and female mice exhibited equal levels of allodynia as male mice. The hypothesis that in female mice allodynia is mediated preferentially by T-cells was tested using T-cell deficient nude and Rag1−/− mice. Both male and female nude and Rag1−/−mice were highly responsive to the reverse of allodynia mediated by glial inhibitor. These findings show that female mice adopt the male, glial-dependent pathway when there is a lack of adaptive immune cells (Sorge et al., 2015a).

7 Cytokines

Cytokines are a broad class of proteins with a small size (~5–25 kDa). These proteins are constitutively expressed on cells membranes in precursor form and can be quickly released by cleaving them thanks to specific enzyme. After the cleavage, they can influence the activity of cells located on a short distance. Even though they are produced by different types of cells, the name that originally referred to them, interleukin (IL), is due to the knowledge that many are synthesized by leukocytes and function on leukocytes. They can be divided into two subclasses, based on their function: These days, it is evident that cytokines have two opposing phenotypes: pro-inflammatory (IL-6, IL-15,IL-1β, TNF) and anti-inflammatory (IL-10, IL-4). Cytokines can exert their role in different way, through direct or indirect activation of targets (Austin & Moalem-Taylor, 2010).

Most of the time pro-inflammatory cytokines play their algesic effects indirectly, through the production of other mediators such as NO and prostaglandin E2, which activate nociceptors. Topical injection of TNF and IL-1 in a skin preparation induce the potentiation of heat-evoked calcitonin gene related peptide (CGRP) release, which promotes nociception by boosting

neurogenic inflammation (Oprée & Kress, 2000), is a notable example of this. On the other hand, there is significant evidence that certain cytokines have direct effects on nociceptors through binding to their receptors (Sommer & Kress, 2004; Spicarova et al., 2011). Moreover, it was shown that TNFα enhance glutamate release in spinal cord dorsal horn in a model of sciatic nerve transection inducing increased spontaneous activity of dorsal horn neurons (Spicarova et al., 2011). Additionally, some cytokines can act via positive feedback. This is the case of TNF, IL-1, and IL-6 inducing each other's production, acting synergistically to amplify inflammatory signals (Watkins et al., 1999), which may contribute to chronic inflammation if not properly blocked.

In order to ensure a well-balanced immune response, anti-inflammatory cytokines act as negative feedback regulators. IL-10 is an example of negative feedback regulation since it decreases the expression of pro-inflammatory cytokine genes, acting on two points, their translation, and down-regulation of their receptors (Strle et al., 2001). As a result, balancing of pro- and anti-inflammatory cytokines is highly responsible for the comprehensive result of human chronic pain states. As consequence, patients with complicated regional pain syndrome show systemic elevations in pro-inflammatory cytokines while anti-inflammatory cytokines are decreased (Davies et al., 2007; Uçeyler, Eberle, et al., 2007). In contrast to this, patients with painless neuropathy have high anti-inflammatory cytokines (Uçeyler, Rogausch, et al., 2007). Thus, cytokine signaling regulation could be investigated as therapy methods for persistent neuropathic pain (Austin & Moalem-Taylor, 2010).

8 Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (**MIF**) is a pro-inflammatory cytokines with chemokine-like functions which play an important role in promoting monocyte recruitment and immobilization (Tillmann et al., 2013). MIF was first identified more than a half-century ago as a T-cell-derived component that inhibited the random pro-inflammatory movement of macrophages out of capillary tubes and was so called macrophage migration inhibitory factor (Bloom & Bennett, 1966; David, 1966)

MIF is present in almost all of the tissues of the body. It is expressed not only in the immune system, taking part as key modulator of both innate and adaptive immune responses, but also in peripheral and central nervous system. In PNS, its expression was shown in sciatic nerve axons and DRG neurons (Alexander et al., 2012). MIF mRNA and protein levels in the brain have been found in astrocytes as well as neurons from the hypothalamus, cortex, pons and cerebellum (Bacher et al., 1998). MIF mRNA was shown to be most abundant in cell bodies, and MIF protein was found in neurons endings (Chalimoniuk et al., 2006). In spinal cord, MIF was found in dorsal horn neurons, axons, microglia, oligodendrocytes and astrocytes but not in ventral horn motor neurons (Alexander et al., 2012). MIF expression is significantly higher in spinal microglia three days after spinal cord injury (SCI) (Koda et al., 2004) (Su et al., 2016) Following compression-induced spinal cord damage, MIF deletion reduces neuronal death and enhances functional recovery in mice (Nishio et al., 2009; Su et al., 2016). Several investigations have shown that monocytes and macrophages are one of the main sources of MIF synthesis and secretion in response to microbial or cytokines stimulation (Mitchell et al., 2002; Su et al., 2016) and MIF itself is able to enhance the release of pro-inflammatory cytokines like TNF alfa (Calandra et al., 1994).

The main receptor for MIF is **CD74**, a type II transmembrane protein (Leng et al., 2003; Zhou et al., 2018). Their binding causes phosphorilation and recruitment of CD44 (Shi et al., 2006), which leads to ERK1/2 activation. Thanks to these downstream actions in the inflammatory cascade, MIF is involved in a variety of diseases which have as common and starting feature the inflammation process such as alzheimer, cancer and neuropathic pain (Grieb et al., 2010). In a formalin-induced hyperlagesia model, MIF expression was increased in a time-dependent manner compared with sham group in spinal cord dorsal horn, cerebro-spinal fluid (CSF) but not in plasma. The same expression pattern was verified for CD74 in SCDH and CSF (F. Wang et al., 2010). Moreover, using fluorescence colocalization, it was demonstrated that microglia cells are the only source of MIF in the inflammatory model. However, in infiltrated spinal T-cells, MIF was not upregulated when compared with the contralateral side (F. Wang et al., 2010). Most of these experiments were repeated in a CCI model and MIF upregulation was found in CSF and SCDH. Differences in CCI and formalin model were found in the fluorescence colocalization experiments, where CD74 expression was increased in microglia cells and MIF expression was increased in neurons, 14 days after the induction of the model. The conclusion is that in a peripheral neuropathic pain model, MIF is produced in spinal neurons and its target might be microglia (F. Wang et al., 2011).

The involvement of MIF in modulating nociceptive behaviors was demonstrated exploiting CCI model and injecting intrathecally MIF antibody and MIF inhibitor, (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester, **ISO-1** (F. Wang et al., 2011). ISO-1 is one of MIF inhibitors with the highest affinity, binds MIF tautomerase active site and blocks MIF-CD74 binding. The prevention of hyperalgesia in mechanical pain test and hypersensitivity to heat stimulus followed almost the same extent in antibody or inhibitor treatments (F. Wang et al., 2011). Thanks to Wang investigations it is clear that one of the cause for peripheral nerve injury-induced neuropathic pain is the elevated levels of spinal MIF, and that the neuropathy can be inhibited by intrathecal administration of MIF tautomerase inhibitor, which functions similarly to MIF antibody.

9 The Endocannabinoid System

The endocannabinoid system (ECS) is composed by three main components: the ligands, called endogenous cannabinoids, anandamide (AEA) and 2-acylglycerol (2-AG); the receptors, cannabinoid receptor 1 and cannabinoid receptor 2 (CB_1 and CB_2); and the enzymes which contribute to the andocannabinoids synthesis and degradation. The ECS exerts its function through the influence of neuronal activity and network function in mature nervous system and its development (Lu & Mackie, 2021).

The ECS regulates several functions, including learning and memory, brain plasticity, nociception, inflammation, neuronal development, hunger management, metabolism, digestion, motility, energy balance, and mood and stress regulation (Matei et al., 2023). Because of the several functions which the ECS regulates, it is critical to recognize that the majority ofits components are multifunctional. As a result, the ECS does not work like an isolated system, but interacts with a wide range of signaling pathways (Matei et al., 2023).

The ECS is composed by two main receptors, cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB2). Both receptors are G protein–coupled receptors (GPCRs) coupled with inhibitory G proteins. CB_1 receptor is primarily found presynaptically on many glutamatergic, GABAergic, serotonergic, cholinergic, and noradrenergic neurons in the CNS. CB_1 is involved in modulating synaptic transmission plasticity. The CB_1 receptor is primarily expressed in the brain in motor and primary sensory regions, as well as cognition, memory, and emotion areas,

neuroendocrine system and autonomic nervous system sections (Kano et al., 2009). CB_2 receptors are mostly found in immune cells (Galiègue et al., 1995; Munro et al., 1993) including microglia (Cabral et al., 2015; Stella, 2010), but they can also be found in neurons (Spiller et al., 2019), especially in pathological situations (Atwood & Mackie, 2010).

9.1 Endogenous cannabinoids

The two most researched endogenous cannabinoids (eCBs), Anandamide (AEA) and 2-arachidonylglycerol (2-AG), are produced by neurons both centrally and peripherally to influence synaptic activity and plasticity (Raichlen et al., 2012). AEA is a fatty acid neurotransmitter that easily crosses the blood-brain barrier and has two main molecular targets, CB_1 and CB_2 receptors (Dietrich & McDaniel, 2004).

There are numerous synthetic mechanisms for creating eCBs, and their relevance varies possibly in some disease conditions, as well as across tissues and development. The classic mechanism for producing 2-AG is a two-step process that begins with the removal of inositol triphosphate from arachidonoyl-containing phosphatidylinositol bisphosphate (PIP2), followed by the removal of the acyl group at the 1 position by a diacylglycerol lipase (Kohnz & Nomura, 2014). The typical process for AEA generation is through N-acyl phosphatidylethanolamine (NAPE) phospholipase D (NAPE-PLD) hydrolysis of NAPE (Jung et al., 2012). Another mechanism to synthetize AEA is through the reverse activity of fatty acid amide hydrolase (FAAH), which usually converts AEA into arachidonic acid and ethanolamine, reduce the availability of AEA into the synaptic cleft and decrease CB receptor activation (Ss et al., 2013).

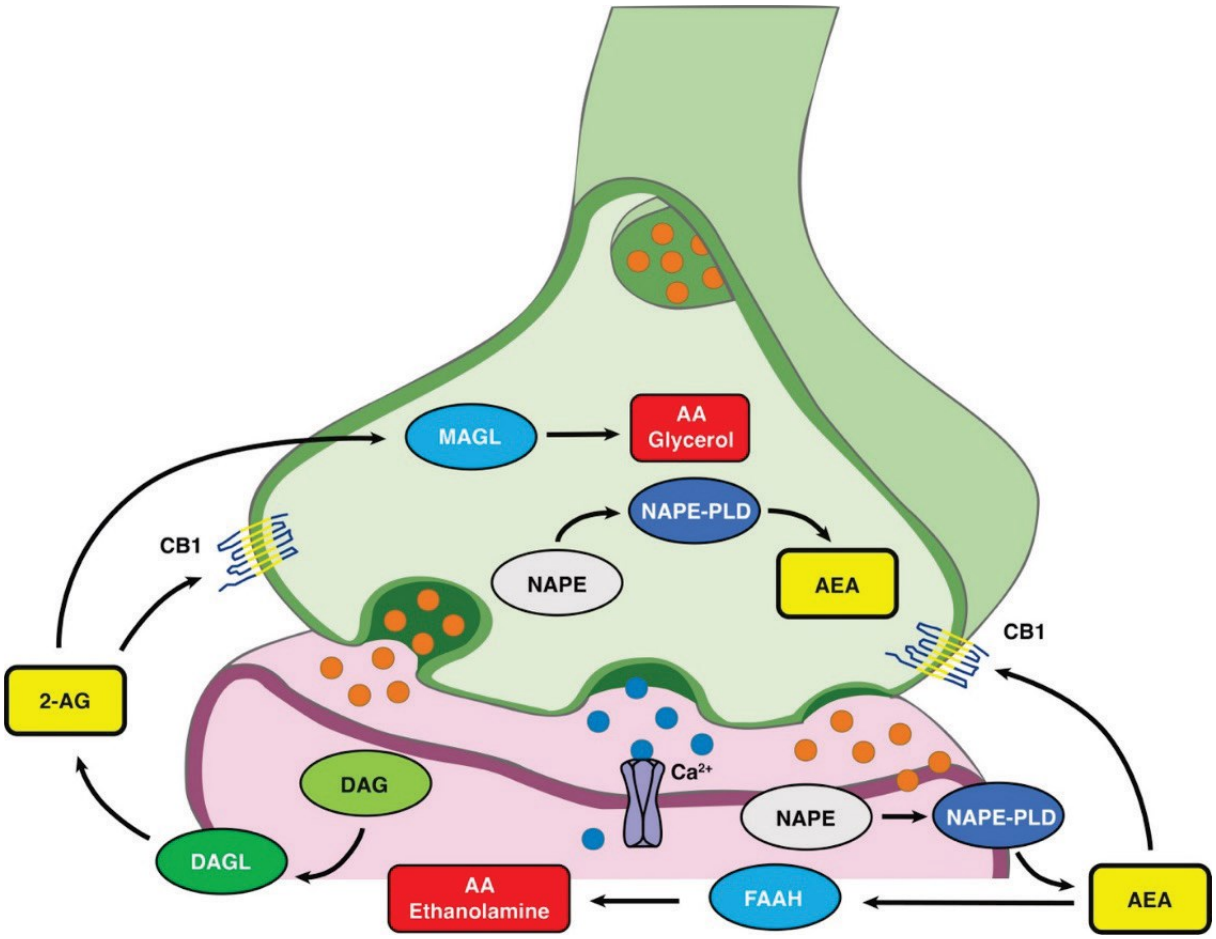

Fig 9.1. Synthesis and degradation of endogenous cannabinoids (Reprinted from Pařízek et al., 2023)

9.2 The Role of Anandamide at the spinal cord level

In previous studies, it was demonstrated that the ECS with all of its components is able to produce antihyperalgesic or antinociceptive effects in several animal models of pain. The binding of endogenous and exogenous cannabinoids to the respective receptor (CB_1) induces the antinociceptive activity through to the inhibition of adenylyl cyclase activity (Felder et al., 1995), consequent inhibition of Ca2+ currents (Twitchell et al., 1997), and modulation of different K+ currents (Mackie et al., 1995) in various site of the CNS. Cannabinoids, in general, diminish glutamatergic transmission in the brain (Shen et al., 1996). They are also implicated in regulation of neurons excitability and firing control (Schweitzer, 2000; Shen et al., 1996). Immunohistochemistry studies have shown labelling of CB_1 receptor in the dorsal horn and dorsal root ganglia suggesting that the spinal cord is a significant location for antinociceptive

activity exerted by the ECS. This finding was supported by behavioral investigations involving intrathecal injection of cannabinoid receptor agonists (Mao et al., 2000), electrophysiological extracellular recordings (Hohmann et al., 1995) and inhibition of C-fiber mediated neurotransmitter release in the spinal cord (Drew et al., 2000).

The role of AEA at spinal cord level seems to be quite fascinating since it has a dual effect. As a matter of fact, the primary AEA receptor is CB_1 receptor and patch clamp recordings from substantia gelatinosa neurons have shown a reduction in the frequency of miniature excitatory postsynaptic currents (m) at 1mM concentration, as expected. But, at concentrations higher of 10mM, AEA induced an increase in the frequency of mEPSC. This effect might be explained by binding of AEA to transient receptor potential vanilloid 1 (TRPV1) receptor, which is a non-selective cation channel (Morisset et al., 2001). While the anandamide-induced reduction in the mEPSP frequency was blocked by SR141716A, a selective cannabinoid receptor antagonist, this compound did not affect the high concentration-induced increase in the frequency. This latter effect of anandamide was completely blocked, however, by ruthenium red, a vanilloid VR1 receptor channel blocker (Morisset et al., 2001).

9.3 Cannabinoid receptor 1 interaction with Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting protein 1

CB_1 receptor activation stimulates various signaling pathways, including G protein-coupled receptor kinases 2/3 (GRK2/3) (W. Jin et al., 1999), which facilitates phosphorylation of CB_1 receptor C-termini at the site of serine/threonine residues (C. A. C. Moore et al., 2007). This causes β-arrestin to be recruited to the GPCR, triggering a cascade of events that drives the receptors to endocytosis, consequent desensitization and development of tolerance (Hájková et al., 2016). SGIP1 is highly expressed in the CNS and PNS, and abundantly present in areas adjacent to presynaptic boutons (Wilhelm et al., 2014). SGIP1 has a significant impact on events that are driven by CB1R C-tail phosphorylation and would result in clatrin mediated endocytosis (CME). The association between CB1R and GRK3 is modulated by SGIP1, having a significantly greater impact on their transitory interaction in later stages of desensitization (Gazdarica et al., 2022). Arrestin-CB1R interaction is also increased in the presence of SGIP1 and lasts shorter when there is a lack of SGIP1 (Hájková et al., 2016). In absence of SGIP1 protein, the interaction between phosphorylated CB1R and arrestins terminates as the receptor

is internalized. SGIP1 interferes with CB1r internalization and, as a consequence, interactions between desensitized CB1R and GRK2/3 and arrestin persist longer in the presence of SGIP1 (Hájková et al., 2016).

10 Aims of the Ph.D. study

The main aim of my Ph.D. project was to study the modulation of nociceptive signalling at the spinal cord level under pathological pain conditions. The focus was on pain-related neuroinflammatory processes and the role of the key nociceptive receptors in peripheral neuropathy and peripheral inflammation.

The first aim was to study the role of proinflammatory cytokine MIF in nociceptive signalling following peripheral neuropathy induced by chronic constriction injury of the sciatic nerve.

The following experimental goals were investigated:

- The effect of MIF inhibitor, ISO-1, on CCI-induced hyperalgesia and gender-related differences.
- The role of systemic administration of ISO-1 in the modulation of excitatory and inhibitory postsynaptic currents recorded from superficial dorsal horn neurons in a neuropathic pain state.
- The role of systemic ISO-1 administration on macrophages migration at the site of injury and in DRGs in a peripheral neuropathy model.
- Regulation of gene expression of cytokine MIF, CCL2, main MIF receptor CD74, macrophages markers CD68 and CD206 by ISO-1 in the spinal cord and DRG in both sexes after CCI injury.

The second aim was to investigate the role of AEA in the modulation of excitatory synaptic transmission at nociceptive synapses in the superficial spinal cord dorsal horn after carrageenan-induced peripheral inflammation.

In particular:

- The effect of CB1, TRPV1 and FAAH inhibition in the AEA-mediated modulation of excitatory postsynaptic currents recorded from superficial dorsal horn neurons in neuroinflammatory conditions.

The third aim was to study the modulatory role of the CB_1 receptor regulatory protein, SGIP1, at first nociceptive synapses of the pain pathway in inflammatory conditions.

Specifically:

- The effect of WIN application on synaptic transmission in SCDH in SGIP1 wild-type and knock-out mice.

11 Methods

11.1 Statement of Ethical Consideration

The local Institutional Animal Care and Use Committee approved all experiments, which were in accordance with the International Association for the Study of Pain guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were developed to minimise distress for animals and the number of animals required for statistical analysis.

11.2 Animals

Male P19–P21 rats were used for spinal cord slice electrophysiology. Adult male mice C57BL/6 weighting 25 to 30 g were used for immunohistochemistry experiments. Adult male and female mice C57BL/6 weighting 25 to 30 g were used for behaviour and PCR experiments. Adult male transgenic mice VGAT-ChR2-eYFP line 81 and SGIP1 -/- were used for patch clamp experiments.

The animals were housed in separate clear plastic cages with soft bedding, free access to food and water, and maintained on 12 hours light/12 hours dark cycle at room temperature controlled conditions. The experiments were carried out during the light phase of the cycle.

11.3 Spinal cord slice preparation

Juvenile Wistar rats and adult mice were used to obtain acute spinal cord slices, following a similar procedure already illustrated in previously published papers (Spicarova & Palecek, 2009). Adult male transgenic mice VGAT-ChR2-eYFP line 8, also known as B6.Cg-Tg(Slc32a1-COP4pH134R/EYFP)8Gfng/J were used for the behavioral, pharmacology, and patch-clamp electrophysiological experiments. Under deep anaesthesia with 3% isoflurane (Forane®, Abbott), laminectomy was performed, and the lumbar spinal cord was removed and immersed in an oxygenated ice-cold dissection solution containing (in mM) 95 NaCl, 1.8 KCl, 7 MgSO4, 0.5 CaCl2, 1.2 KH2PO4, 26 NaHCO3, 25 D-glucose, and 50 sucrose. The spinal cord was then fixed to vibratome stage (Leica VT1200S, Germany) using cyanoacrylate glue in a groove between two agar blocks. Transverse slices 300 μm thick were cut from the lumbar segment L3-L5, incubated in the dissection solution for 30 min at 33 °C and then stored in a recording solution at room temperature until used for the electrophysiological experiments. The recording solution contained (in mM): 127 NaCl, 1.8 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, 25 D-glucose. For the actual measurement, slices were transferred into a recording chamber continuously perfused with the recording solution at a rate ~ 2 ml/min. All extracellular solutions were saturated with carbogen (95 % O2, 5 % CO2) during the whole process.

11.4 Patch-clamp recording

Spinal cord slices were used for patch-clamp recordings from superficial dorsal horn neurons in lamina I and II (outer). A differential interference contrast (DIC) microscope (Leica, DM LFSA, Germany) equipped with a near-infrared-sensitive camera (Hitachi KP-200P, Japan) with a standard TV/video monitor was used to visualize individual neurons. Borosilicate glass tubing was used to prepare patch pipettes with resistances ranging from 3.5 to 6.0 MΩ once

filled with intracellular solution. The intracellular pipette solution was composed by (in mM): 125 gluconic acid lactone, 15 CsCl, 10 EGTA, 10 HEPES, 1 CaCl2, 2 MgATP, 0.5 NaGTP and was adjusted to pH 7.2 with CsOH. Voltage-clamp recordings in the whole-cell configuration were performed with an Axopatch 200B amplifier and Digidata 1440A digitizer (Molecular Devices, USA) at room temperature (~ 23 °C). Whole-cell recordings were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. The series resistance of neurons was routinely compensated by 80% and was monitored during the whole experiment. AMPA receptor-mediated spontaneous EPSCs were recorded from neurons in the presence of 10 μM bicuculline and 5 μM strychnine. Based on the composition of the intracellular and extracellular solution, a liquid junction potential was calculated using pClamp 10.5 Software and correction implemented; thus, recordings of EPSC were performed at -85 mV. Miniature EPSCs (mEPSC) were distinguished by the addition of 0.5 μM tetrodotoxin (TTX) to the bath solution. Optogenetic approach to record the light-evoked (le) inhibitory postsynaptic currents (IPSC) and distinguish between excitatory and inhibitory neurons was used (Adamek et al., 2022). In this study, only excitatory neurons without ChR2-mediated plateau phase after a stimulation with blue light (470 nm) of 500-ms-length were included. Glycine receptor- and/or GABAAR-mediated sIPSC and le-IPSC were recorded using the same recording and intracellular solution at 0 mV. AMPA receptor antagonist CNQX (20 mM) and NMDA blocker AP5 (25 mM) were used to record IPSC. For data acquisition and for off-line analysis, Software package pCLAMP 10.5 (Axon Instruments, USA) was used.

11.5 Animal models

11.5.1 Carrageenan model

Peripheral inflammation was induced under 3% isoflurane anesthesia. To induce peripheral inflammation in mice, a 1% mixture of carrageenan in a physiological solution was used, and 3% mixture of carrageenan with saline was used in ~P20 rats. Carrageenan was injected subcutaneously to both hind paws. Mechanical paw withdrawal threshold (PWT) was tested before carrageenan injection (on Day 0), and after on Day 1 (~24 h later), before spinal cord slices preparation and patch-clamp experiment. Naive animals were used as controls.

11.5.2 Chronic constriction injury model

CCI was performed in adult mice under 3% isoflurane anesthesia. Three loose ligatures were tied proximal to the trifurcation of the sciatic nerve. PWT was tested before (Day 0) and after CCI on Days 1, 3, and 7 before spinal cord slices preparation and patch-clamp experiment. PWT and paw withdrawal latency (PWL) were tested before (Day 0) and after CCI on Days 3, 7, 9, 11, 14, 21 and 28. Naive animals were used as controls.

11.6 Drug treatment

All basic chemicals, used for the preparation of the dissection, recording and intracellular solution, were of analytical grade and purchased from Sigma-Aldrich (Prague, Czech Republic) and Tocris Bioscience (Bristol, UK). ISO-1 was dissolved in DMSO, which had a concentration of < 0.1 % in the final solution. ISO-1 (Tocris) stock solution (200 mM) was prepared freshly in DMSO (Sigma-Aldrich) just before the treatment. Based on the weight of the mice, an appropriate dose of ISO-1 (20 mg/kg i.p.) was mixed with the vehicle (saline to reach 5% final concentration). ISO-1 was injected one hour before every surgery and everyday until day 6.

11.7 Behavior tests

11.7.1 Paw withdrawal threshold to mechanical stimulation

To perform behavioural tests, mice were placed on a stainless steel we mesh under clear acrylic glass cages in a quiet room and allowed to acclimate for ∼1 hour. Paw withdrawal threshold (PWT) to tactile stimulation was tested manually in the morning hours (8:00 to 11:00 AM). Control PWT was tested in all groups on Day −3 and 0 before any treatment. We used electronic von Frey apparatus (IITC Life Sciences, Model 2390 Series, USA). The probe tip of electronic von Frey was applied 4 times to the plantar surface. The average value from each hind paw was calculated and then averaged in the experimental group. A quick flick or full paw withdrawal was considered a response. The averaged numbers of responses on each bending force were calculated for each hind paw and then averaged in the experimental group. All data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism was used for statistical

analysis. Data from experiments with the electronic von Frey apparatus were analyzed by Two Way ANOVA (treatment × time) with Tukey post hoc test.

11.7.2 Time of Reaction to thermal stimulation

The adult mice used for the preparation of the peripheral inflammation model were tested to thermal stimuli before and 24 hours after the model induction. Time of Reaction (TR) to heat stimuli was measured for both hind paws using the Hot Plate apparatus. The temperature is set to 53°C± 0.2. On the testing day, mice are placed on the surface of the hot plate and covered by a glass transparent cylinder. A 30 second cut-off time is assigned in this protocol. The latency to response is recorded when the first hind paw lick or jump occurs. Each response was tested 4 times with at least 5 min between the trials. The averaged values from the hind paws of individual animals were averaged in the experimental groups. Data from experiments with Hot Plate apparatus were analyzed by Two Way ANOVA (treatment × time) with Tukey post hoc test.

11.8 Immunohistochemistry

11.8.1 Study of CD68-immunoreactive macrophages in sciatic nerve and DRGs

Adult male mice C57BL/6 were randomly distributed in three experimental groups: control (CTRL group, n = 3); chronic constriction injury (CCI group, n = 3); chronic constriction injury + ISO-1 (CCI + ISO-1 group, n = 3). Animals were 1 h later deeply anaesthetized with a combination of ketamine (100 mg/kg, Narketan, Zentiva) and xylazine (25 mg/kg, Xylapan, Zentiva), perfused intracardially with saline followed by ice-cold 4% paraformaldehyde. Both L5 DRGs and sciatic nerves were removed and post-fixed in 4% paraformaldehyde at 4° C for 2 h, cryoprotected with 30% sucrose overnight, and cut in cryostat Leica CM3000 to 16 μm thick slices. Every 3rd DRG section was then processed for CD68 immunohistochemistry. Briefly, sections were washed 3× for 10 min in phosphate-buffer solution (PBS), blocked with 3% normal donkey serum (NDS) for 30 min at room temperature, and incubated overnight at 4° C with rabbit anti-CD68 primary antibody (1:200, Abcam, #ab125212) in 1% NDS with 0.3% Triton X-100. After washing in 1% NDS (3× for 10 min), the sections were exposed to a donkey anti-mouse AlexaFluor-488 secondary antibody (1:400, Jackson ImmunoResearch Laboratories, #711-545-152) for 2 h. For visualization of the cell nucleus, incubation in

bisbenzimide (Hoechst 33342, Sigma-Aldrich) for 3 min was used. Slices were mounted by DPX mounting medium. Pictures were captured using a confocal microscope (Leica Microsystems, SP8). Multi-immersion objective HC PL APO (20x/0.75 NA), Ar multiline laser (488 nm; 65 mW), and HyD spectral detectors were used. Data were analyzed offline using ImageJ software (National Institutes of Health) by an investigator blinded to the treatment, using ROI containing only neuronal cell bodies area (excluding nerve/root fibers). CD68 IR area was analyzed in ROI using the Threshold function. Left and right L5 DRGs were analysed separately (CTRL: n = 6, CCI group: n = 6, CCI+ISO-1 group: n = 6). Longitudinal sections from the site of the injury moving proximal and distal to the spinal cord were made. Slices of the sciatic nerve were divided in segments, 4 segments for the proximal and 4 segments for the distal part.

11.8.2 Study of CD206-immunoreactive macrophages in sciatic nerve and DRGs

Adult male mice C57BL/6 were randomly distributed in three experimental groups: control (CTRL group, n = 3); chronic constriction injury (CCI group, n = 3); chronic constriction injury + ISO-1 (CCI + ISO-1 group, n = 3). Animals were perfused, and tissue was collected and processed (as described above) 24 h after the last treatment. Rabbit anti-CD206 primary antibody [ED1] (1:200, Abcam, #ab64693) and a donkey anti-mouse AlexaFluor-488 secondary antibody (1:400, Jackson ImmunoResearch Laboratories, #715-545-151) were used. Slices were mounted by DPX mounting medium. Pictures were captured using a confocal microscope (Leica Microsystems, SP8). Multi-immersion objective HC PL APO (20x/0.75 NA), Ar multiline laser (488 nm; 65 mW), and HyD spectral detectors were used. Data were analyzed offline using ImageJ software (National Institutes of Health) by an investigator blinded to the treatment, using ROI containing only neuronal cell bodies area (excluding nerve/root fibers). CD206 IR area was analyzed in ROI using the Threshold function. Left and right L5 DRGs were analyzed separately (CTRL: n = 6, CCI group: n = 6, CCI+ISO-1 group: n = 6). Longitudinal sections from the site of the injury moving proximal and distal to the spinal cord were made. Slices of the sciatic nerve were divided in segments, 4 segments for the proximal and 4 segments for the distal part.

11.9 PCR

At day 3 after the CCI, the animals were deeply anesthetized and tissues of interest were removed, frozen in liquid nitrogen and stored at −80°C. The total RNA from the spinal cord dorsal horn and DRGs was isolated with the commercially available kit RNeasy Mini (Qiagen) according to the manufacturer's protocol. Concentration and purity of total RNA were determined by measurements of the absorbance at 260 and 280 nm using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies). Samples were treated with DNase (RNase-free DNase set, Qiagen) to avoid the contamination with genomic DNA. cDNA was synthetized using High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative PCR was carried out using a Viia 7 Real-Time PCR System (Applied Biosystems), 5× Hot Firepol Probe QPCR Mix Plus (ROX) (Solis BioDyne) and premade TaqMan Assays (Thermo Fisher Scientific) specific for the studied transcript. The following assays were used: CCL2 (ThermoFisher, #4453320), CD74 (ThermoFisher, #4331182), CD68 (ThermoFisher, #4331182), CD206 (ThermoFisher, #4331182), MIF (ThermoFisher, #4453320). GAPDH was chosen as a proper housekeeping gene for our experiments. Fold differences of relative mRNA levels over vehicle control were calculated by $2^{-\Delta\Delta}$CT method.

12 Results

12.1 MIF antagonist ISO-1 attenuates the development of neuropathic pain in a model of peripheral neuropathy

12.1.1 ISO-1 effect on the hypersensitivity induced by peripheral neuropathy in male and female mice

In our behavioral experiments, we investigated the effect of systemic administration of MIF inhibitor ISO-1 on hypersensitivity induced by CCI in rodents of both sexes. The concentration of ISO-1 (20 mg/kg) to inject intraperitoneal was based on a previous study (Alexander et al., 2012).

Only male mice were used in the first set of experiments, and the early period of allodynia was studied. The CCI induced strong mechanical hypersensitivity, and the Paw Withdrawal Threshold (PWT) decreased already one day after the surgery (Day 0: 8.4 ± 0.6 g, Day 1: 4.6 ± 0.8 g, n=10, $p < 0.001$, Figure 12.1) and still decreased three days after the surgery (3.8 ± 0.3 g, n=10 $p < 0.001$). ISO-1 was used as a pretreatment (1h before CCI) and on the first and second day after the surgery (20 mg/kg, n=9, Fig 12.1). The MIF inhibitor did not change PWT at each tested time points, Day 1 and Day 3. In conclusion, in early stages of neuropathic pain model, ISO-1 did not affect the CCI-induced mechanical allodynia in males.

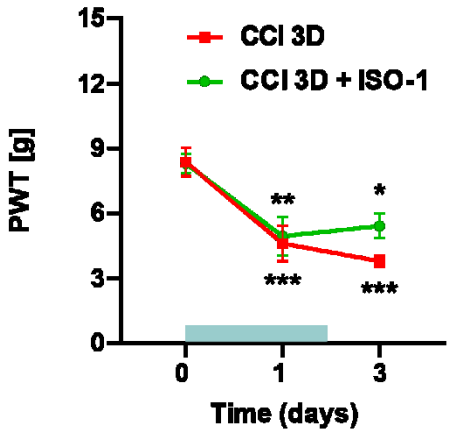

Figure 12.1. Mechanical hypersensitivity induced by CCI was not affected by the MIF inhibitor, ISO-1, short-term treatment. CCI significantly reduced PWT from the 1st to the

3rd day after the surgery (n=10). Systemic administration of ISO-1 (i. p., 20 mg/kg every day from Day 0 to Day 2, n=9) did not change the CCI-induced allodynia on Day 1 and Day 3. Statistical analysis: *p < 0.05, **p < 0.01, ***p < 0.001 versus control before CCI (Day 0), Mixed-effects model followed by Tukey post hoc test.

In the second series of our experiments, we wanted to explore the role of ISO-1 (i. p., 20 mg/kg every day from Day 0 to Day 6) in the later stages of CCI-induced mechanical and thermal hypersensitivity. In electronic Von Frey, CCI decreased significantly PWT at Day 3 (Day 0: 8.2 ± 0.2 g, Day 3: 3.9 ± 0.2 g, n=14, p<0.0001, fig 12.2) and Day 7 (3.6 ± 0.2 g, p<0.0001, fig 12.2). The PWT increased and returned close to control values 7 days after CCI and ISO-1 long-term treatment (Day 0: 8.1 ± 0.2 g, Day 7: 7.3 ± 0.4 g, n=13, p<0.001, fig 12.2).

In Hot Plate test, CCI reduced significantly the Time of Reaction (TR) to the heat stimulus on the third (Day 0: 9.7 ± 0.4 s, Day 3: 3.5 ± 0.3 s, n=5, p< 0.01, Figure 12.2) and seventh day after the surgery (3.9 ± 0.3 s, n=5, p< 0.01, Figure 12.2). ISO-1 treatment affected the TR increasing it significantly at Day 7 (Day 3: 4.6 ± 0.4 s, Day 7: 9.1 ± 0.3 s, n=6, p< 0.001, Figure 12.2). To conclude, in later stages of neuropathic pain model, ISO-1 treatment attenuated the CCI-induced mechanical and thermal hypersensitivity.

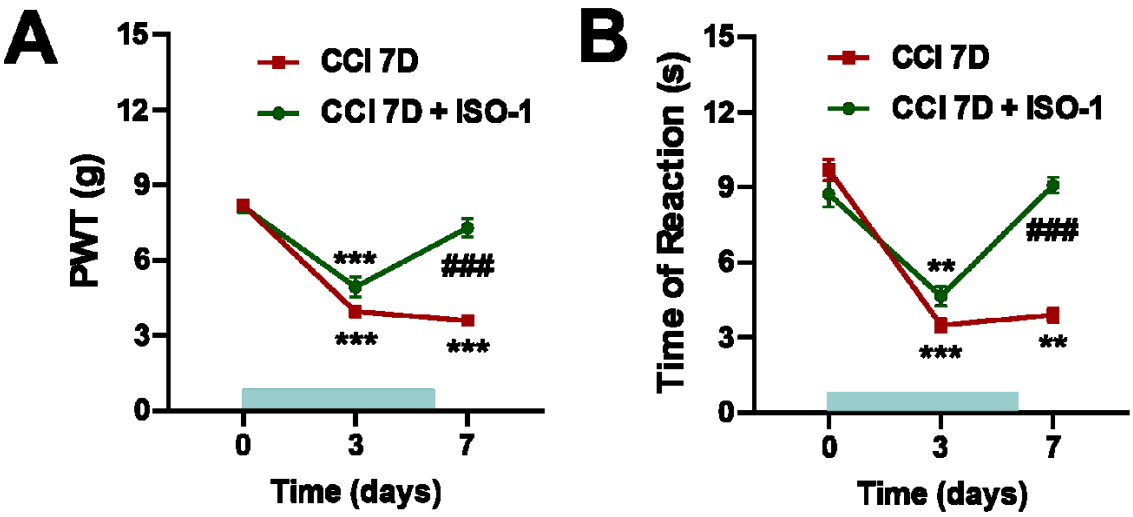

Figure 12.2. Mechanical and thermal hypersensitivity induced by CCI was reversed by the MIF inhibitor, ISO-1, long-term treatment seven days after the surgery in male.

(A) CCI reduced PWT from the 3rd to the 7th day after the surgery in male mice (n=14). ISO-1 treatment (i.p., 20 mg/kg, every day from Day 0 to Day 6, n=13) significantly prevented the CCI-induced mechanical hypersensitivity 7 days after CCI surgery in males. **(B)** CCI decreased the TR on Hot Plate test 3 and 7 days after CCI in males (n=5). ISO-1 treatment significantly increased the TR on Hot Plate test on day 7 after the CCI surgery in males. Statistical analysis: **p < 0.01, ***p < 0.001 versus control before CCI (Day 0), ### p < 0.001 versus CCI group, Mixed-effects model followed by Tukey post hoc test.

In order to investigate ISO-1 effect once the model of peripheral neuropathy is well established on both sexes, we decided to add a third set of behavior experiments, in which ISO-1 treatment (i.p., 20 mg/kg, everyday from Day 0 to Day 6) was stopped at the 7th day after the CCI. Mechanical and thermal hypersensitivity were tested for a 28 days after injury interval in males and females. In male mice, CCI significantly decreased PWT from Day 3 (Day 0: 7.7 ± 0.2 g, Day 3: 3.5 ± 0.1 g, n=5, p<0.001, fig 12.3) and this effect lasted for 28 days after the surgery (Day 28: 3.4 ± 0.09 g, n=5, p<0.001, fig 12.3). ISO-1 treatment (i.p., 20 mg/kg, from Day 0 to Day 6) was able to significantly attenuate the CCI-induced mechanical allodynia at Day 7 (7.3 ± 0.3 g, n=6, p<0.001, fig 12.3) and this effect was protracted until Day 9 (5.9 ± 0.2 g, n=6, p<0.001). In Hot Plate test, the TR was significantly reduced from Day 3 (Day 0: 9.7 ± 0.4 s, Day 3: 3.5 ± 0.3 s, n=5, p<0.001, fig 12.3) and this effect lasted for the whole 28 days period (Day 28: 3.01 ± 0.2 s, p<0.001, fig 12.3). MIF inhibitor (i.p., 20 mg/kg, every day from Day 0 to Day 6) significantly increased the TR from Day 7 (9.1 ± 0.3 s, n=6, p<0.001, fig 12.3) until Day 14 (9.1 ± 0.5 s, n=6, p<0.001, fig 12.3).

In female mice, CCI significantly reduced PWT (Day 0: 8.1 ± 0.1 g, Day 3: 3.7 ± 0.3 g, n=5, p<0.001, fig 12.3) and TR (Day 0: 8.9 ± 0.6 s, Day 3: 3.6 ± 0.3 s, n=5, p<0.001, fig 12.3) from Day 3 and the effect was prolonged for the 28 days after the injury. ISO-1 treatment (i.p., 20 mg/kg, every day from Day 0 to Day 6) did not affect PWT neither TR at every time point tested. To conclude, MIF inhibition by ISO-1 treatment attenuated CCI-induced mechanical allodynia and thermal hyperalgesia only in males. Inhibition of thermal hyperalgesia lasted for a longer period than inhibition of mechanical allodynia by the same ISO-1 treatment. In fact, ISO-1 protective effect on mechanical sensitivity lasted for three days once the treatment was stopped, meanwhile for thermal hypersensitivity the protective effect of MIF inhibitor lasted for 7 days after stopping the treatment.

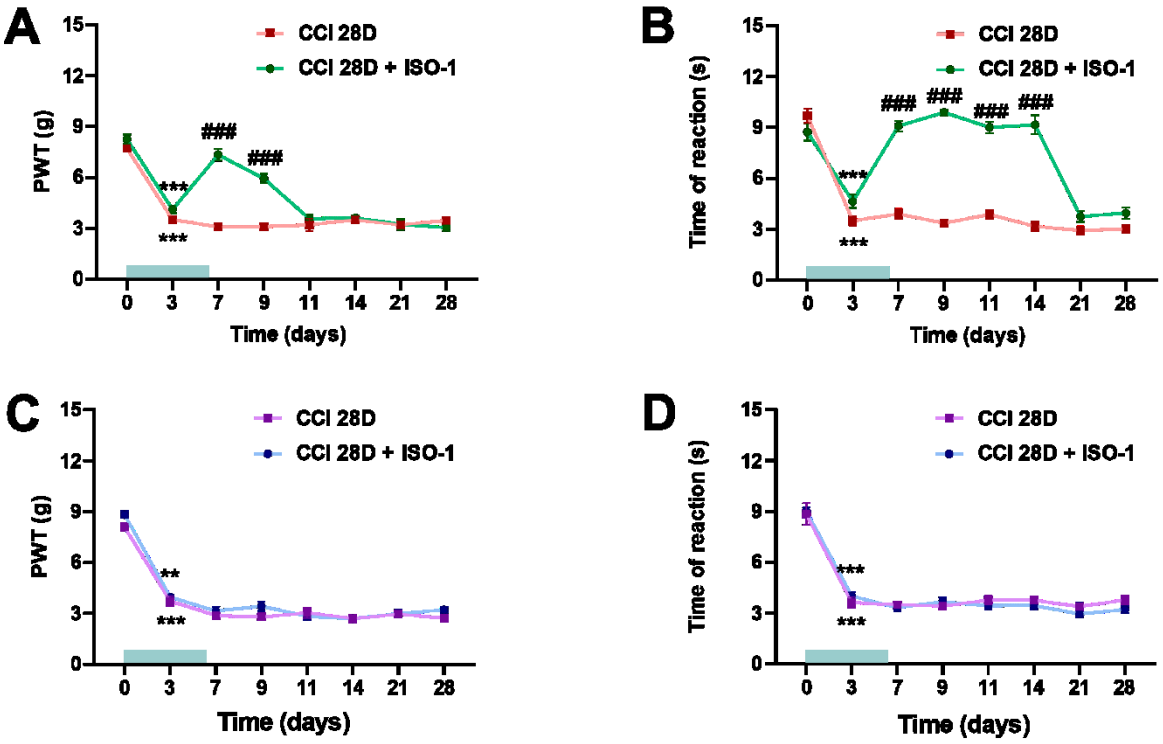

Figure 12.3. 7 days ISO-1 treatment effect on mechanical and thermal sensitivity in both sexes. (A) CCI reduced significantly PWT in male from the 3rd day after the surgery; ISO-1 (i.p., 20 mg/kg, every day from Day 0 to Day 6) reverted this effect and the inhibition lasted from Day 7 until Day 9. **(B)** CCI significantly decreased the TR in male from the 3rd day after the injury. ISO-1 did increase TR in male and this effect lasted from Day 7 until Day 14. (C) and (D) PWT and TR in female mice significantly decreased from Day 3 after the CCI. ISO-1 did not effect both mechanical and thermal hypersensitivity in females. Statistical analysis: ** $p < 0.01$ and ***$p < 0.001$ versus control before CCI (Day 0), ### $p < 0.001$ versus CCI group. Mixed-effects model followed by Tukey post hoc test.

12.1.2 CCI-induced changes in synaptic transmission at the spinal cord level were modulated by ISO-1

We performed whole-cell patch clamp experiments to investigate the modulation of nociceptive synaptic transmission by ISO-1. Specifically, we studied MIF inhibitor effect on the disruption of the balance between excitatory and inhibitory transmission at the SCDH level caused by the peripheral neuropathy It was already demonstrated that pathologic pain may disrupt excitatory and inhibitory neurotransmission, leading to an enhancement of the excitatory and decrease of

the inhibitory synaptic transmission (Zeilhofer et al., 2012). In our experiments, three experimental groups of animals (CTRL, CCI and CCI+ISO-1) were used to record spontaneous postsynaptic currents (sEPSC), spontaneous inhibitory postsynaptic currents (sIPSC), and light-evoked inhibitory postsynaptic currents (le-IPSC) in superficial spinal cord neurons.

In the first set of experiments, we focused on the excitatory synaptic transmission. In the control animals, sEPSC frequency has a mean value of 1.82 ± 0.28 Hz, meanwhile 7 days after CCI, sEPSC frequency increased to 5.47 ± 0.7 Hz ($p<0.001$, n=20, Figure 12.4). Intraperitoneal treatment of neuropathic animals with ISO-1 (i.p., 20 mg/kg, every day from Day 0 to Day 6)decreased significantly sEPSC frequency to 2.9 ± 0.5 Hz ($p< 0.01$, n=16, Figure 12.4) when compared to the CCI group values. The amplitude of the sEPSCs did not change for the whole recording (CTRL: 19.9 ± 1.9 pA, CCI: 14.9 ± 1.1 pA, CCI + ISO-1: 19.9 ± 1.2 pA). These results indicate that sciatic nerve injury enhanced spontaneous excitatory synaptic transmission in superficial dorsal horn neurons, and this potentiation was prevented by ISO-1 treatment.

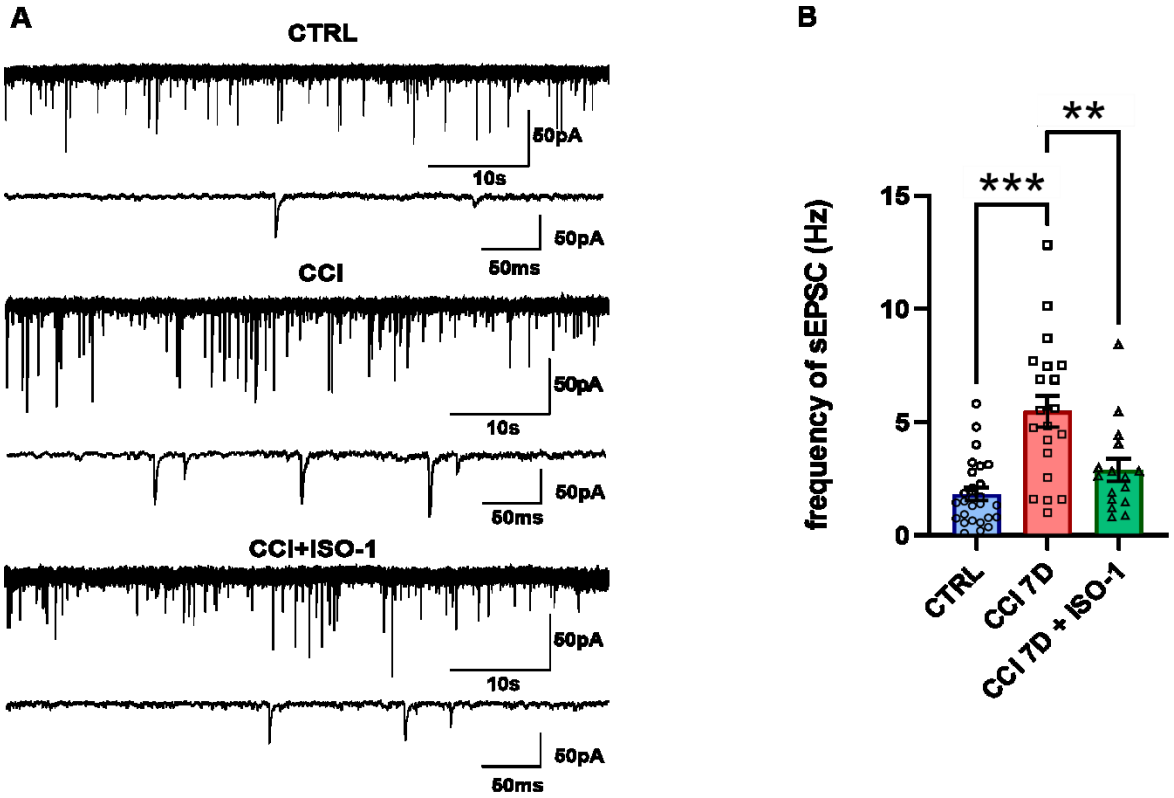

Figure 12.4. Systemic ISO-1 administration decreased sEPSC frequency in a CCI model. **(A)** One minute (top) and one second (bottom) traces of native recordings of sEPSCs in three experimental groups from one superficial dorsal horn neuron. **(B)** The frequency of sEPSC was increased 7 days after CCI excitatory dorsal neurons recorded (n=20). ISO-1 i.p. treatment (20 mg/kg, every day from Day 0 to Day 6) significantly decreased the CCI-induced increase of

sEPSC frequency (n=16). Statistical analysis: **p < 0.01, ***p < 0.001, one-way ANOVA followed by Tukey's multiple comparisons test.

In the second set of experiments, we focused on inhibitory synaptic transmission. In the same experimental groups (CTRL, CCI and CCI+ISO-1), we recorded sIPSC following the sEPSC recording period. sIPSCs frequency in control animals has a mean value of 1.31 ± 0.2 Hz and CCI reduced the frequency significantly to 0.66 ± 0.1 Hz (p < 0.05, n=18, Figure 12.5). ISO-1 treatment did not change sIPSC frequency on day 7 after injury when compared with injury without the ISO-1 treatment. The amplitude of the sIPSCs did not change for the whole recording (CTRL: 19.3 ± 1.9 pA, CCI: 17.3 ± 1.6 pA, CCI + ISO-1: 20.5 ± 2.4 pA). These data suggest that sciatic nerve injury decreased spontaneous inhibitory synaptic transmission in superficial dorsal horn neurons, and systemic application of ISO-1 did not influence this loss of inhibition, disinhibition, caused by the peripheral neuropathy.

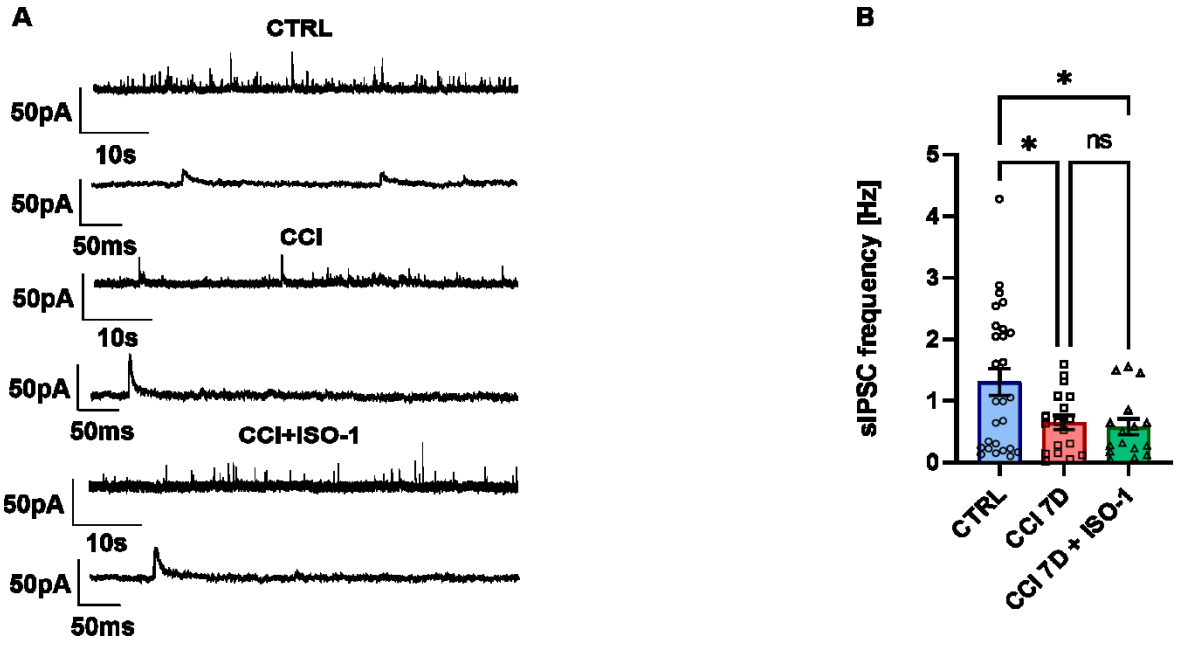

Figure 12.5. Systemic ISO-1 administration did not affect sIPSC frequency in a CCI model. (A) One minute (top) and one second (bottom) traces of native recordings of sIPSCs in three experimental groups from one superficial dorsal horn neuron. **(B)** The frequency of sIPSC was decreased 7 days after CCI in excitatory dorsal horn neurons recorded (n=18). ISO-1 i.p. treatment (20 mg/kg, every day from Day 0 to Day 6) did not change the CCI-induced decrease

of sIPSC frequency (n=16). Statistical analysis: *p < 0.05, one-way ANOVA followed by Tukey's multiple comparisons test.

In the third set of experiments, we use optogenetic approach to investigate the le-IPSCs focusing on inhibitory synaptic transmission as well. By photostimulation (5ms, 470nm) we analysed the amplitudes of le-IPSC in the same groups of neurons. In the control group (n=28) of excitatory dorsal horn neurons, the mean le-IPSC amplitude was 793.9 ± 74.5 pA. Injury to the sciatic nerve (CCI 7D group) decreased le-IPSC amplitude to 491.6 ± 52.6 pA (p < 0.05, n=19, Figure 12.6). ISO-1 treatment (i.p., 20mg/kg, every day from Day 0 to Day 6) significantly increase le-IPSC amplitudes to 910.8 ± 147.7 pA when compared with le-IPSC amplitudes from CCI group neurons (p < 0.05, n=16, Figure 12.6). These results show that ISO-1 prevented CCI-induced le-IPSC amplitude inhibition. To conclude, MIF inhibitor was able to restore excitatory transmission back to a control situation but it did not affect the spontaneous inhibitory transmission. On the other hand, ISO-1 restored amplitudes of le-IPSC.

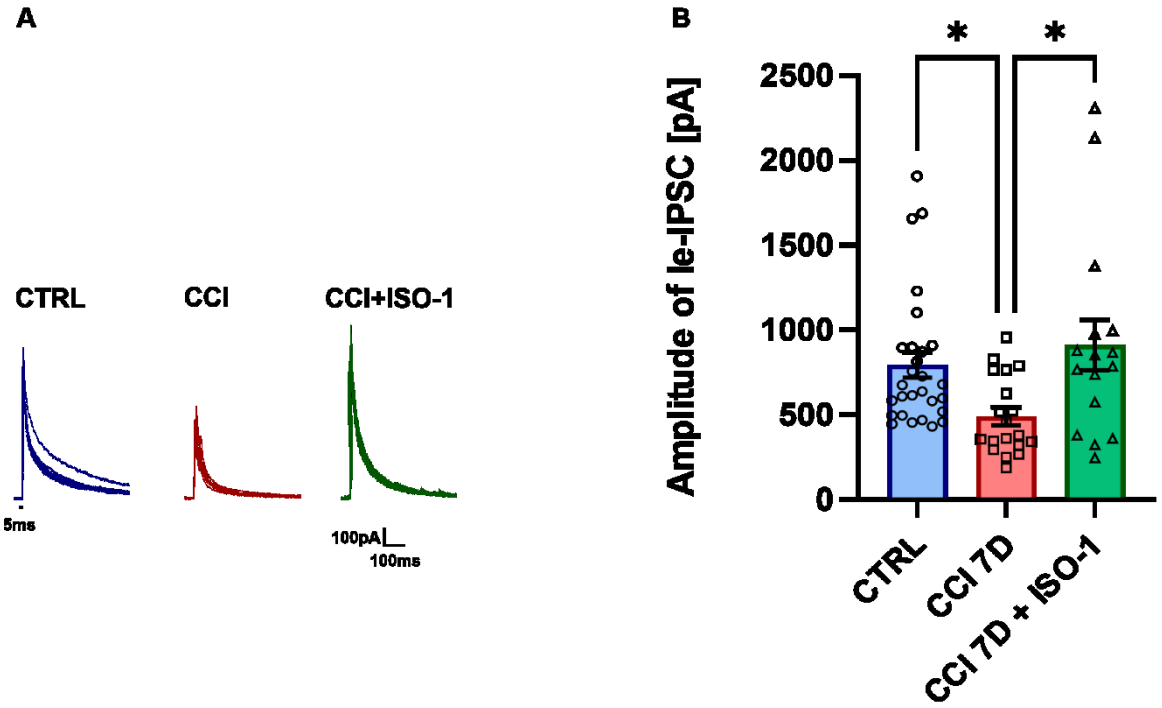

Figure 12.6. Systemic ISO-1 administration increased le-IPSC amplitudes in a CCI model. **(A)** Traces of native recordings of le-IPSCs in excitatory dorsal horn neurons from the control (CTRL) group of mice, on day 7 after sciatic nerve injury (CCI) and CCI with ISO-1 i.p.

treatment (CCI+ISO-1). **(B)** was 7 days after CCI induced decrease of le-IPSCs amplitudes on day 7 after injury(n=20). ISO-1 i.p. treatment (20 mg/kg, every day from Day 0 to Day 6, n=16) increased the CCI-induced decrease of le-IPSCs amplitudes to the control level. Statistical analysis: *p < 0.05, one-way ANOVA followed by Tukey's multiple comparisons test.

12.1.3 MIF inhibitor treatment reduced CCI-induced infiltration of CD68 and CD206 immunoreactive macrophages in sciatic nerve and DRGs

It is well known that ISO-1 acts on MIF activity (Lubetsky et al., 2002) and one of the main MIF functions is to enhance the cytokines release after an inflammatory stimulus. Therefore, cytokines will act on activation and migration of immune cells, among them macrophages are involved in the initial stage of the neuroinflammatory response.

ISO-1 treatment affected CCI-induced increased CD68 immunoreactivity in DRG

We have studied the effect of ISO-1 in vivo treatment (20mg/kg i.p., every day from Day 0 to Day 6) on CD68 positive cells infiltration in L5 DRGs and sciatic nerve sections after the induction of peripheral neuropathy.

In DRGs, immunohistochemistry analysis of CD68 showed that 7 days after CCI of sciatic nerve, CD68 positive area is significantly increased (4.2 ± 0.3%, n=15 $p < 0.001$, fig 12.7) when compared with the CTRL group (1.08%, n=8). ISO-1 treatment significantly decreased the CD68 positive area (0.75 ± 0.1%, n=16 $p < 0.001$, fig 12.7) when compared with CCI group values. The enhanced CCI-induced migration of CD68 positive cells in DRGs was strongly influenced by MIF inhibitor, ISO-1, which restored the immune system involvement back to CTRL situation.

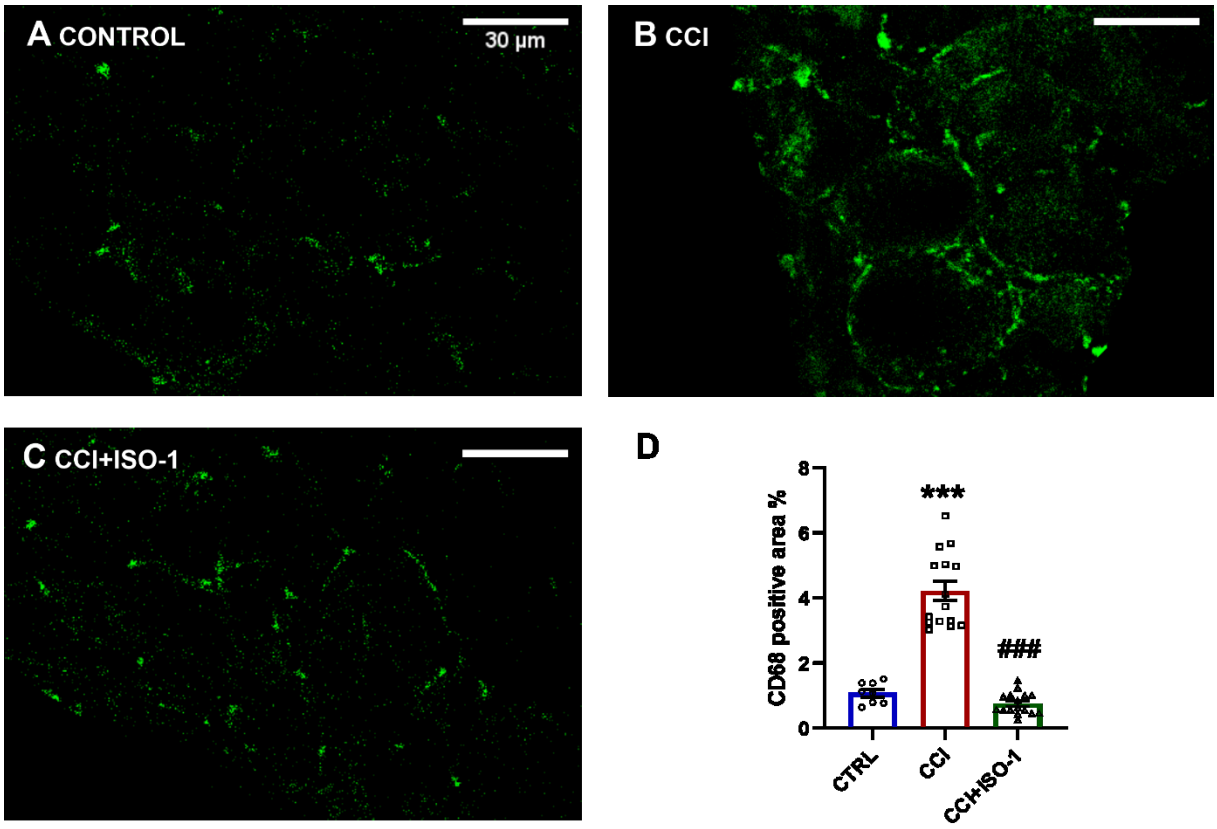

Fig 12.7. Systemic ISO-1 injections prevent the CCI-induced increase of CD68 positive area in DRGs. Representative images of CD68 immunofluorescence in the DRG of the **(A)** control, **(B)** CCI, **(C)** CCI+ISO-1 group. **(D)** Staining of CD68 in L5 DRGs slices shows an increase in CD68 positive area 7 days after CCI surgery. This increase was prevented by ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 6). Statistical analysis: ***$p < 0.001$ vs CTRL. ###$p < 0.001$ vs CCI+ISO-1. Differences between treatments were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

ISO-1 treatment affected CCI-induced increased CD68 immunoreactivity in sciatic nerve

We have investigated CD68 positive macrophages infiltration also in the sciatic nerve. Three experimental groups were used: CTRL, **7** days after sciatic nerve injury (CCI) and CCI with ISO-1 i.p. treatment (CCI+ISO-1). Transversal sections from the site of the injury moving proximal and distal to the spinal cord were made. Transversal slices of the sciatic nerve were divided in segments, 4 segments for the proximal and 4 segments for the distal part. Analysis of P1 segment, the proximal segment closest to the injury, unveiled a significant increase of

CD68 positive area when compared with staining in the control group of animals (CTRL group, 0.76 ± 0.3%, n=6; CCI group, 19.70 ± 1.2%, n=7, $p < 0.001$, fig 12.8). ISO-1 treatment did not affect CD68 positive area after CCI. Analysis of P2 segment, a segment closer to the spinal cord and further from the injury than P1 segment, showed a significant increase of CD68 positive area in CCI group compared with CTRL group (CTRL group, 0.48 ± 0.1%, n=6; CCI group, 15.67 ± 1.6%, n=8, $p < 0.001$, fig 12.8). ISO-1 treatment significantly decreased the area in which CD68 was present (8.2 ± 1.4%, n=8, $p < 0.01$, fig 12.8). At the P3 segment, CD68 immunoreactivity was higher in CCI group compared with CTRL group (CTRL group, 0.45 ± 0.05%, n=6, CCI group, 11.5 ± 1.8%, n=7, $p < 0.001$, fig 12.8) and ISO-1 treatment significantly reduced this effect (3.6 ± 0.7%, n=7, $p < 0.001$, fig 12.8). In the P4 segment, the closest to the spinal cord and furthest from the injury, CD68 immunopositivity was significantly higher in CCI than in CTRL group (CTRL group, 0.46 ± 0.1%, n=6; CCI group, 8.34 ± 1.9%, n=7, $p < 0.001$, fig 12.8). ISO-1 significantly reduced the CD68 immunopositivity (2.2 ± 0.43%, n=8, $p < 0.01$, fig 12.8).

Analysis of D1, the distal segment closest to the injury, unveiled a significant increase of CD68 positive area in CCI compared with CTRL group (CTRL group, 0.9 ± 0.1%, n=6; CCI group, 17.3 ± 2.4%, n=7, $p < 0.01$, fig 12.9). Treatment with MIF inhibitor did not affect CD68 positive area after CCI in D1. CD68 immunoreactivity in D2 segment was significantly higher in CCI than CTRL group (CTRL group, 0.78 ± 0.02%, n=6; CCI group, 20.34 ± 1.6%, n=8, $p < 0.001$, fig 12.9) and ISO-1 significantly decreased the area in which CD68 was present (10.50 ± 2.3%, n=7, $p < 0.01$, fig 12.9). At the D3 segment, CD68 staining was significantly increased in CCI than in CTRL group (CTRL group, 0.63 ± 0.03%, n=6; CCI group, 12.9 ± 1.7%, n=8, $p < 0.001$, fig 12.9) and ISO-1 treatment reduced significantly CD68 staining (6.1 ± 1.4%, n=7, $p < 0.01$, fig 12.9). In D4, the furthest segment to the injury and to the spinal cord, CD68 immunopositivity was significantly higher in CCI than in CTRL (CTRL group, 0.57 ± 0.02%, n=6; CCI group, 12.1 ± 0.8%, n=8, $p < 0.001$, fig 12.9). ISO-1 treatment significantly reduced CD68 immunopositivity (4.8 ± 0.96%, n=7, $p < 0.001$, fig 12.9). In conclusion, blocking MIF tautomerase activity reduced macrophages infiltration to the site of the injury and to the DRGs.

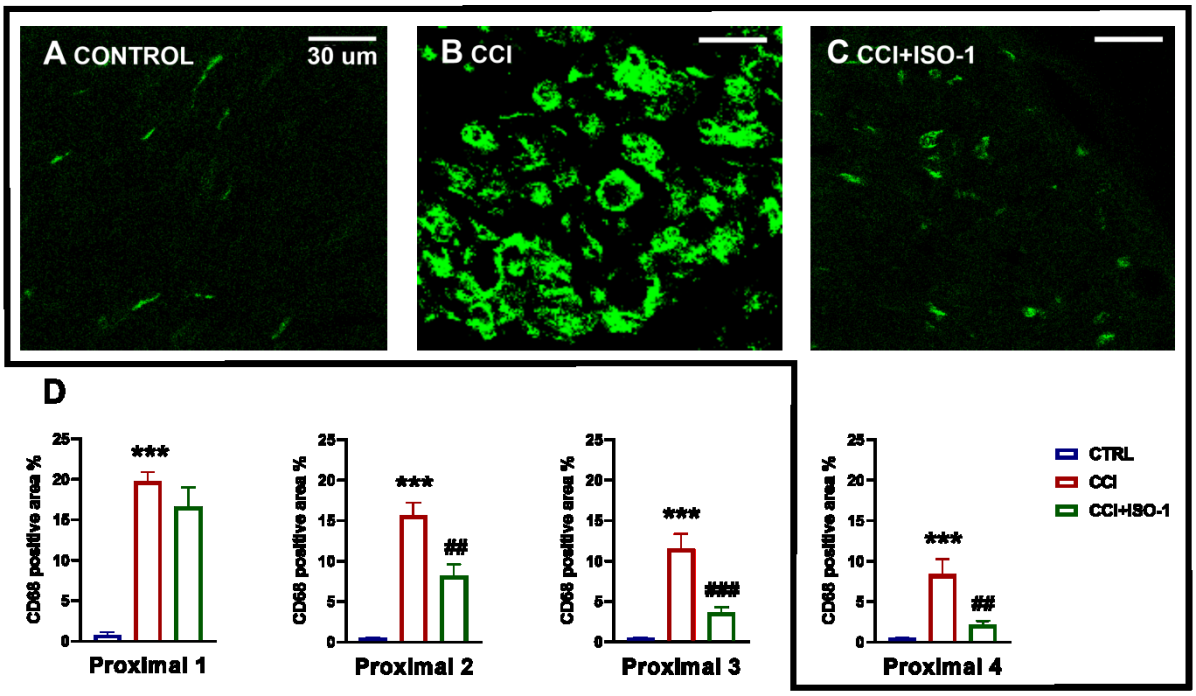

Fig 12.8. Systemic ISO-1 treatment decreased CD68 positive area 7 days after CCI in sciatic nerve proximal segments. Representative images of CD68 immunofluorescence in the sciatic nerve of the **(A)** control, **(B)** CCI, **(C)** CCI+ISO-1 group. **(D)** Staining of CD68 in sciatic nerve transversal slices. Segment 1 is closest to the injury. CCI caused a significant increase in CD68 positive area in all of the segments. This increase was lowered by ISO-1 treatment in the second, third and fourth segment (20 mg/kg i.p., every day from Day 0 to Day 6). Statistical analysis: ***$p < 0.001$ vs CTRL. ##$p < 0.01$, ###$p < 0.001$ vs CCI+ISO-1. Differences between groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

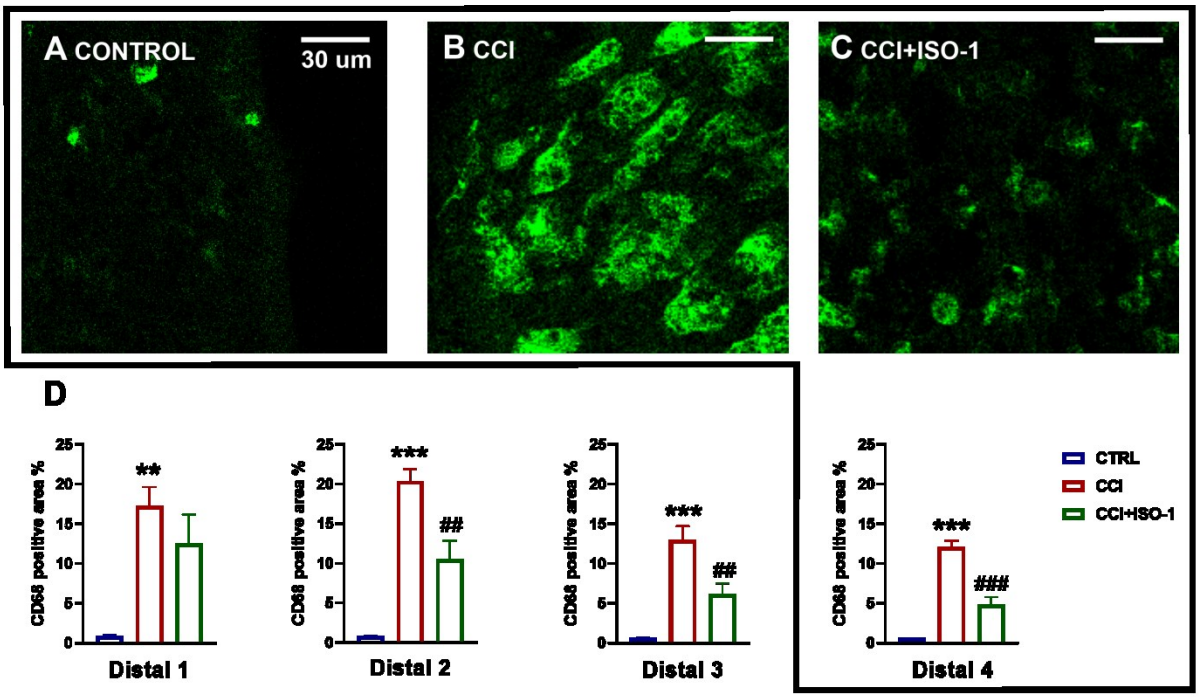

Fig 12.9. Systemic ISO-1 treatment decreased CD68 positive area 7 days after CCI in sciatic nerve distal segments. Representative images of CD68 immunofluorescence in the sciatic nerve of the **(A)** control, **(B)** CCI, **(C)** CCI+ISO-1 group. **(D)** Staining of CD68 in sciatic nerve transversal slices. Segment 1 is closest to the injury. CCI caused a significant increase in CD68 positive area in all of the segments. This increase was lowered by ISO-1 treatment in the second, third and fourth segments (20 mg/kg i.p., every day from Day 0 to Day 6). Statistical analysis: **p < 0.01, ***p < 0.001 vs CTRL. ##p < 0.01, ###p < 0.001 vs CCI+ISO-1. Differences between groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

MIF inhibitor prevented CCI-induced infiltration of CD206 immunoreactive macrophages in DRG

Similar to CD68 study, we have investigated the effect of ISO-1 in vivo treatment (20 mg/kg i.p., every day from Day 0 to Day 6) on CD206 positive cells infiltration in L5 DRGs and sciatic nerve sections after the induction of peripheral neuropathy.

In DRGs, we found that 7 days after CCI of sciatic nerve, CD206 positive area is significantly increased compared with CTRL situation (CTRL group, 6.1 ± 0.2%, n=5; CCI group, 9.8 ± 0.29%, n=17, p < 0.001, fig 12.10). ISO-1 7 days treatment significantly decreased the CD206 positive area (mean= 4.9 ± 0.2%, n=14, p < 0.001, fig 12.10) when compared with values from

CCI group. Immunohistochemistry data of CD206 staining on DRGs showed the effect of ISO-1 in lowering activation and migration of CD206 positive cells towards the DRGs after the sciatic nerve injury.

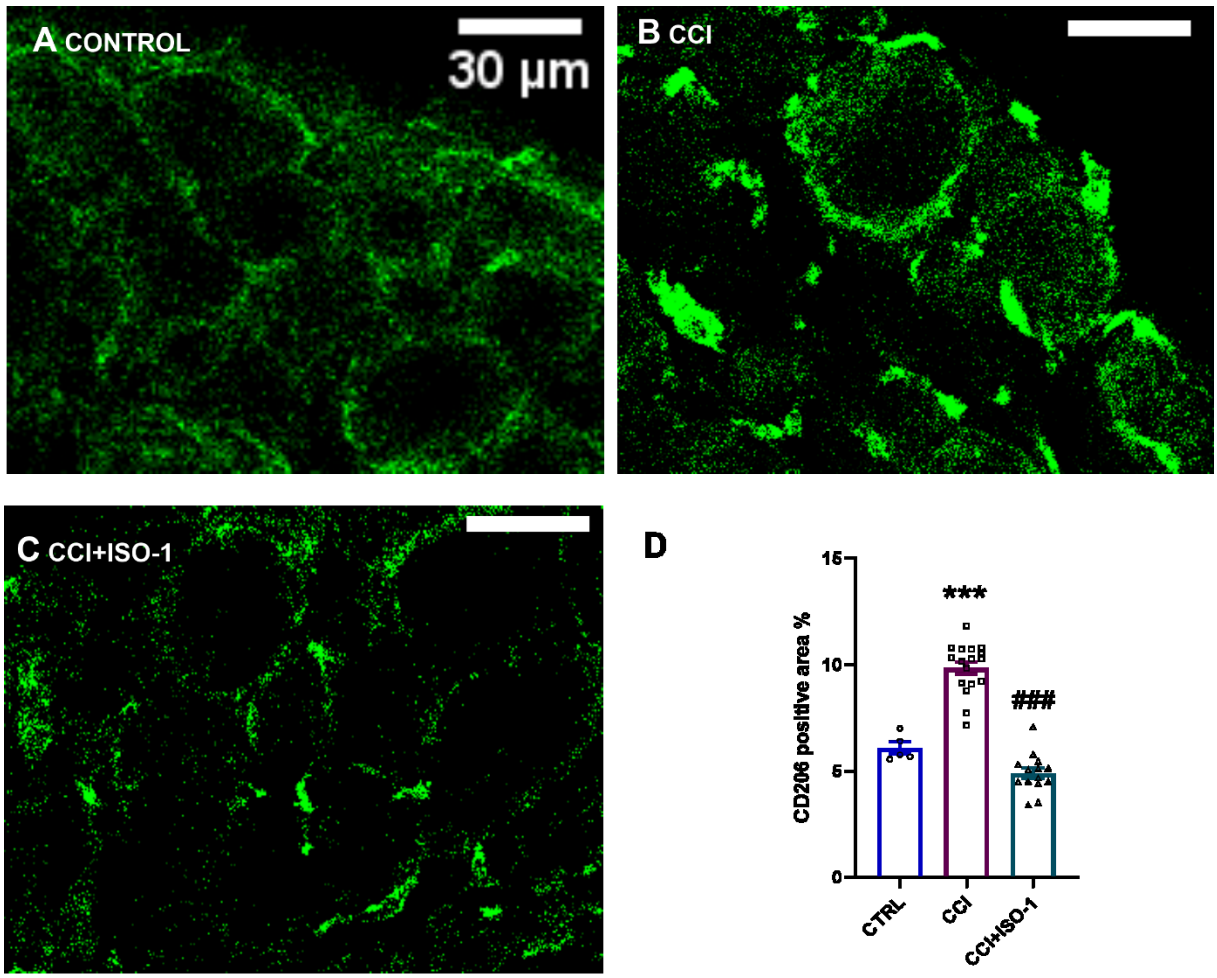

Fig 12.10. Systemic ISO-1 injections decreased CCI-induced infiltration of CD206 immunoreactive macrophages in DRGs. Representative images of CD206 immunofluorescence in the DRG of the **(A)** control, **(B)** CCI, **(C)** CCI+ISO-1 group. **(D)** Staining of CD206 in L5 DRGs slices shows an increase in CD206 positive area in CCI group. This increase was lowered close to CTRL values by ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 6). Statistical analysis: ***$p < 0.001$ vs CTRL. ###$p < 0.001$ vs CCI+ISO-1. Differences between treatments were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

Similar to CD68 macrophages investigation, we have studied CD206 positive macrophages invasion also in the sciatic nerve. Three experimental groups were used: CTRL, **7** days after

sciatic nerve injury (CCI) and CCI with ISO-1 i.p. treatment (CCI+ISO-1). Transversal sections from the site of the injury moving proximal and distal to the spinal cord were made. Transversal slices of the sciatic nerve were divided in segments, 4 segments for the proximal and 4 segments for the distal part. Analysis of P1 segment, the proximal segment closest to the injury, unveiled a significant increase of CD206 positive area when compared with staining in the control group of animals (CTRL group, 1.2 ± 0.2%, n=4; CCI group, 14.35 ± 1.6%, n=5, $p < 0.001$, fig 12.11). ISO-1 treatment did not affect CD68 positive area after CCI. Analysis of P2 segment, a segment closer to the spinal cord and further from the injury than P1 segment, showed a significant increase of CD206 positive in CCI group when compared with CTRL group (CTRL group, 1.2 ± 0.1%, n=4; CCI group, 15.7 ± 1.2%, n=4, $p < 0.001$, fig 12.11). ISO-1 decreased the area in which CD206 was present but this decrease was not significant. At the P3 segment, CD206 immunoreactivity was higher in CCI group compared with CTRL group (CTRL group, mean= 1.2 ± 0.1%, n=4; CCI group, 11.3 ± 1.4%, n=5, $p < 0.001$, fig 12.11) and the decrease induced by ISO-1 treatment was not significant. In the P4 segment, the closest to the spinal cord and furthest from the injury, CD206 immunopositivity was significantly higher in CCI than in CTRL group (CTRL group, 1.2 ± 0.1%, n=4; CCI group, mean= 10.8 ± 0.9%, n-6, $p < 0.001$, fig 12.11). ISO-1 significantly reduced the CD206 immunopositivity (mean= 5.8 ± 0.9%, n=6, $p < 0.01$, fig 12.11).

Analysis of the distal segments, starting from the closest to the injury, D1, unveiled a significant increase of CD206 positive area in CCI compared with CTRL group (CTRL group, 1.4 ± 0.1%, n=4, CCI group, 13.70 ± 1.2%, n=6, $p < 0.001$, fig 12.12). Treatment with MIF inhibitor did not affect CD206 positive area after CCI. Moving distally to the spinal cord, in the D2 segment was detected a significant increase of CD206 positive area in CCI compared with CTRL group (CTRL group, 1.1 ± 0.2%, n=4, CCI group, 14.5 ± 0.95%, n=6, $p < 0.001$, fig 12.12) and ISO-1 significantly decreased the area in which CD206 was present (7.6 ± 1.4%, n=6, $p < 0.01$, fig 12.12). At the D3 segment, CD206 staining was significantly increased in CCI than in CTRL group group (CTRL group, 1.3 ± 0.2%, n=4, CCI group, 12.3 ± 1.5%, n=6, $p < 0.001$, fig 12.12) and ISO-1 treatment was able to reduce significantly this effect, as it is shown by the data (6.2 ± 1.4%, n=6, $p < 0.05$, fig 12.12). In D4, the furthest segment to the injury and to the spinal cord, CD206 immunopositivity was significantly higher in CCI than in CTRL (CTRL group, 1.2 ± 0.2%, n=4, CCI group, 11.6 ± 0.9%, n=6, $p < 0.001$, fig 12.12). ISO-1 treatment significantly reduced CD206 immunopositivity (5.9 ± 1.2%, n=6, $p < 0.01$, fig 12.12). To conclude, ISO-1 was able to influence the level of M2 type macrophages, the anti-inflammatory

subgroup, and to revert CCI-induced increase of M2 macrophages in the DRGs and sciatic nerve.

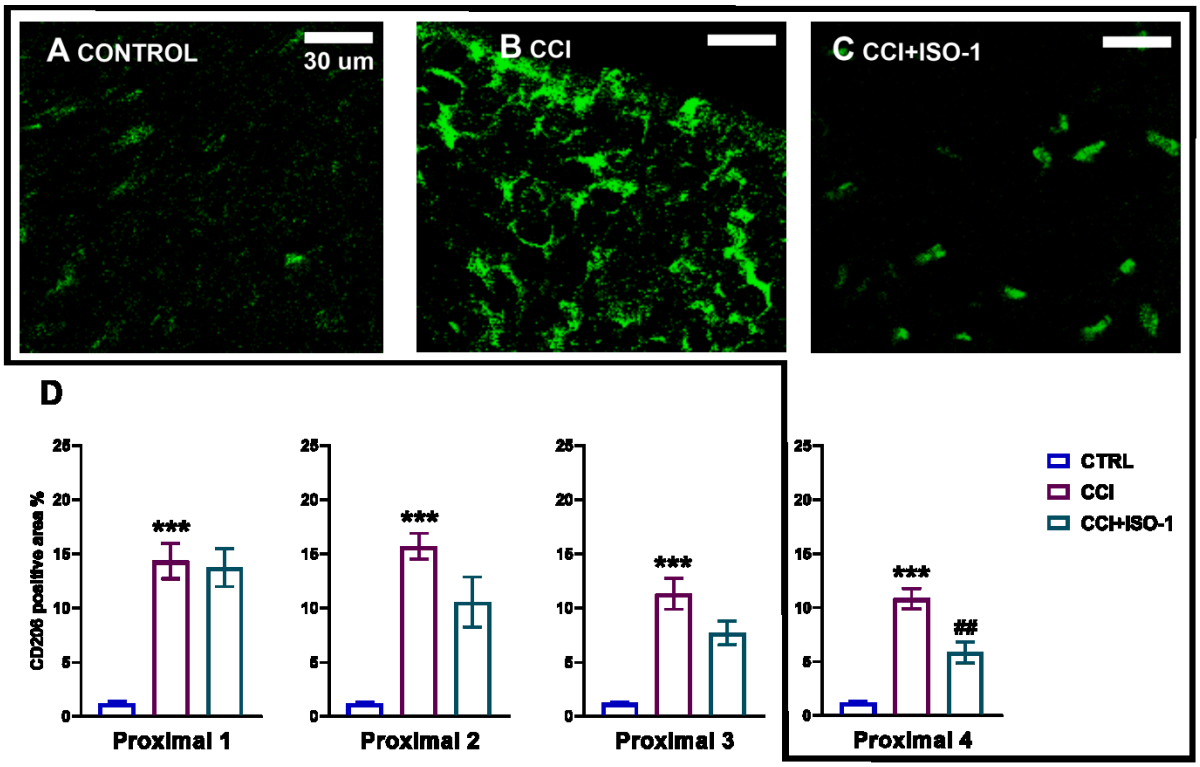

Fig 12.11. Systemic ISO-1 treatment decreased CD206 positive area 7 days after CCI in sciatic nerve proximal segments. Representative images of CD206 immunofluorescence in the sciatic nerve of the **(A)** control, **(B)** CCI, **(C)** CCI+ISO-1 group. **(D)** Staining of CD206 in sciatic nerve transversal slices. Segment 1 is closest to the injury. CCI caused a significant increase in CD206 positive area in all of the segments. This increase was lowered by ISO-1 treatment in the fourth segment (20 mg/kg i.p., every day from Day 0 to Day 6). Statistical analysis: ***$p < 0.001$ vs CTRL. ##$p < 0.01$ vs CCI+ISO-1. Differences between groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

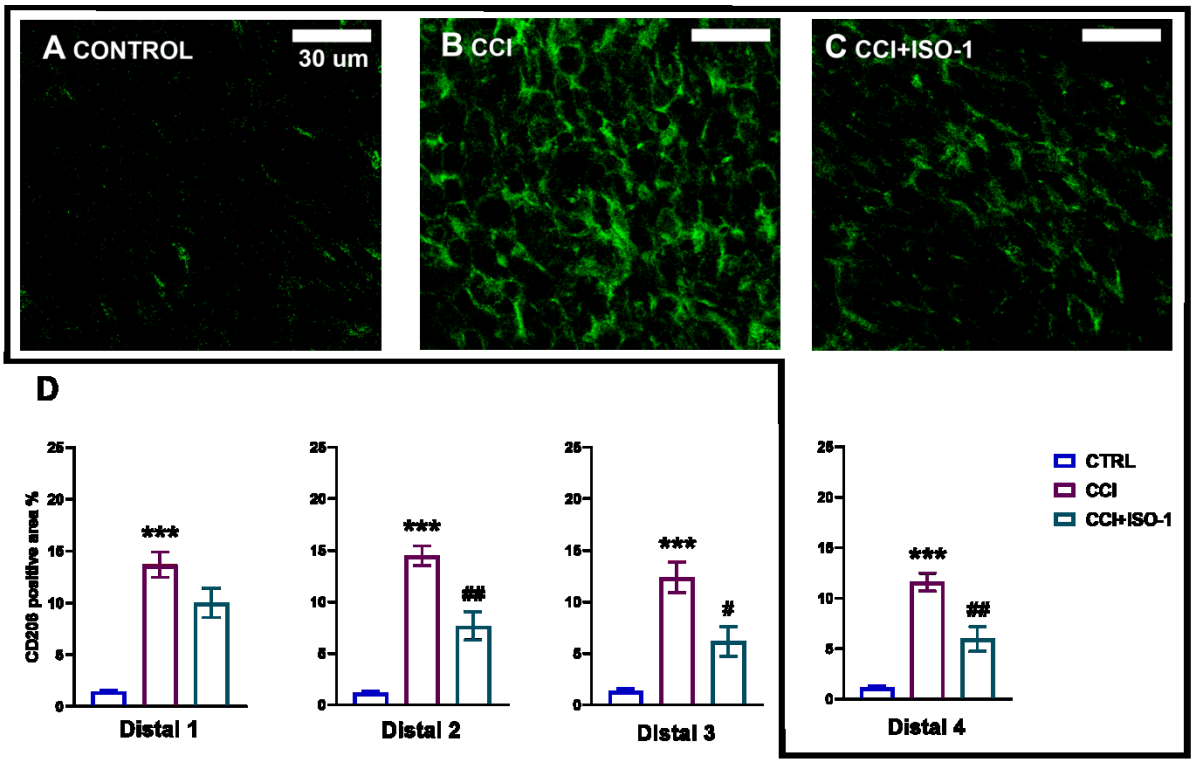

Fig 12.12. Systemic ISO-1 treatment decreased CD206 positive area 7 days after CCI in sciatic nerve distal segments. Representative images of CD206 immunofluorescence in the sciatic nerve of the **(A)** control, **(B)** CCI, **(C)** CCI+ISO-1 group. **(D)** Staining of CD206 in sciatic nerve transversal slices. Segment 1 is closest to the injury. CCI caused a significant increase in CD206 positive area in all of the segments. This increase was lowered by ISO-1 treatment in the second, third and fourth segment (20 mg/kg i.p., every day from Day 0 to Day 6). Statistical analysis: ***$p < 0.001$ vs CTRL. #$p < 0.05$, ##$p < 0.01$ vs CCI+ISO-1. Differences between groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

12.1.4 Inhibition of MIF-CD74 binding plays a different role in modulating signs of CCI-induced neuroinflammation in male and female mice

In order to reveal the modulation of MIF-CD74 pathways mediated by ISO-1 (20 mg/kg i.p., every day from Day 0 to Day 2) in a early phase of neuroinflammation induced by CCI model, we have performed measurements of RNA levels at Day 3 after CCI in both sexes. Specifically, we have performed PCR experiments to study changes in the RNA of five proteins related with MIF activity (MIF, CD74, CCL2, CD68 and CD206) 3 days after CCI and 3 days after CCI and ISO-1 systemic application at the spinal cord (SC) and DRG level.

In males' SC an increase in RNA level of MIF, CD74, CCL2 (CTRL vs CCI: 13.12 ± 2.7, n=6, fig. 12.15), CD68 (CTRL vs CCI: 10.01 ± 3.1, n=6, fig. 12.16) and CD206 (CTRL vs CCI: 1.2 ± 0.27, n=6, fig. 12.17) was shown 3 days after CCI. ISO-1 treatment for three days attenuated RNA expression of MIF, CCL2 (CTRL vs CCI+ISO-1: 3.8 ± 0.3, n=5, $p < 0.05$, fig. 12.15) and CD68 (CTRL vs CCI+ISO-1: 3.5 ± 0.47, n=8, $p < 0.05$, fig. 12.16). On the other hand, ISO-1 treatment increased significantly CD206 RNA expression (CTRL vs CCI+ISO-1: 4.5 ± 0.9, n=8, $p < 0.05$, fig. 12.17). An increase, after ISO-1 treatment, in gene expression was revealed also for CD74 but this increase was not significant.

In females' SC, an increase in RNA level of MIF, CD74, CCL2, CD68 (CTRL vs CCI: 11.4 ± 0.8, n=6, fig. 12.16) and CD206 (CTRL vs CCI: 1.2 ± 0.1, n=6, fig. 12.17) was shown 3 days after CCI. ISO-1 attenuated CCL2 RNA level but this effect was not significant. MIF inhibitor treatment did not affect expression of MIF and CD74 proteins. On the other hand, ISO-1 3 days treatment induced a significant decrease in the pan macrophages marker, CD68, levels (CTRL vs CCI+ISO-1: 5.5 ± 1.5, n=8, $p < 0.01$, fig. 12.16) and an increase of the M2 type macrophages marker, CD206 (CTRL vs CCI+ISO-1: 3.4 ± 0.57, n=8, $p < 0.01$, fig. 12.17). To conclude, ISO-1 had a different effect on the expression of MIF and CD74 in males and females but it affected CCL2, CD68 and CD206 RNA levels following the same trend in both sexes in the spinal cord.

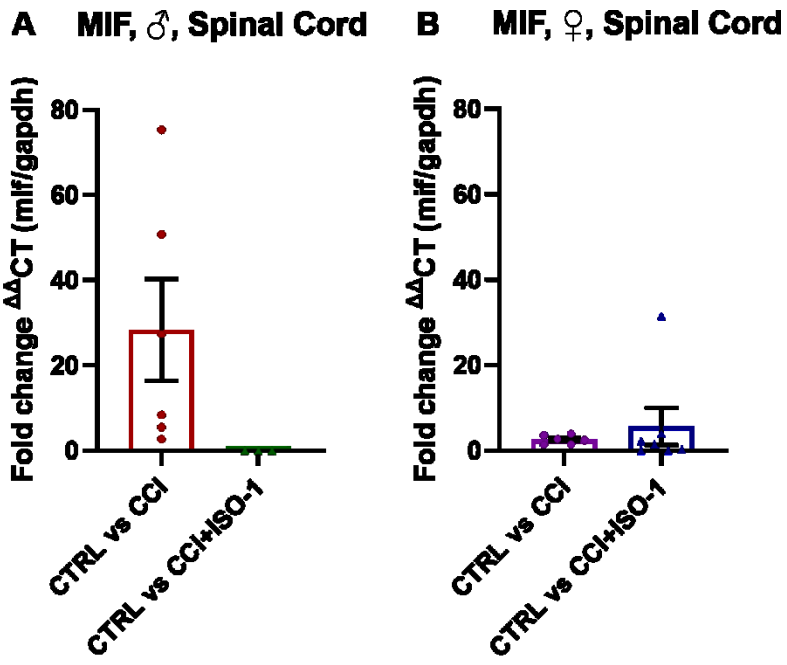

Fig 12.13. ISO-1 effect on MIF gene expression after CCI at the spinal cord level in both sexes. Relative changes in *mif* gene expression normalized to *gapdh* (endogenous control) in SC three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison.

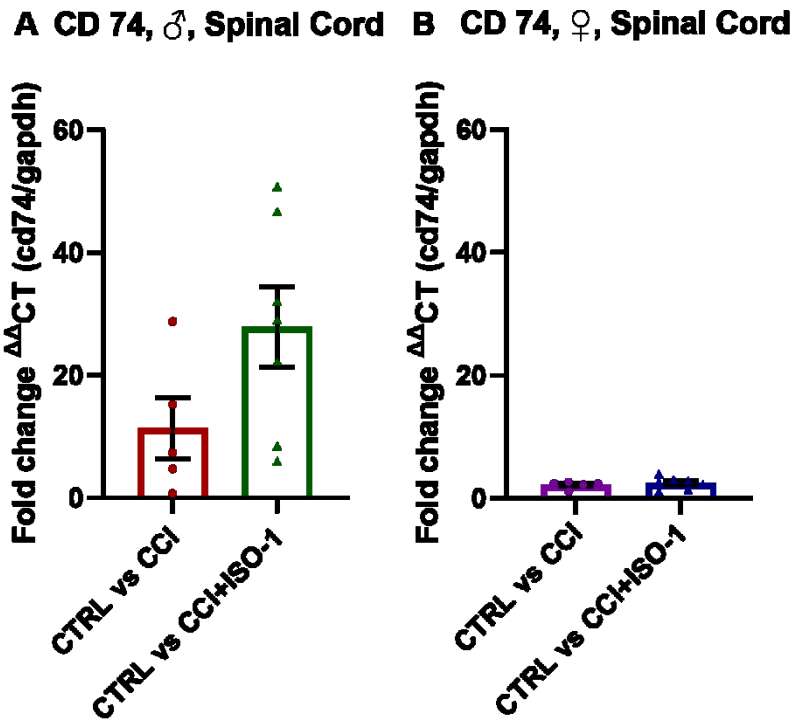

Fig 12.14. ISO-1 effect on CD74 gene expression after CCI at the spinal cord level in both sexes. Relative changes in *cd74* gene expression normalized to *gapdh* (endogenous control) in SC three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison.

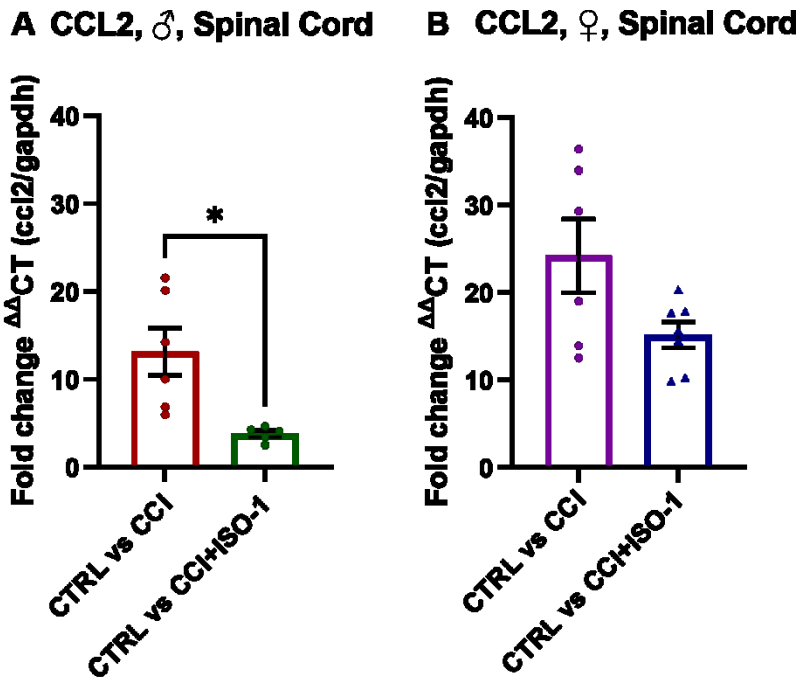

Fig 12.15. ISO-1 effect on CCL2 gene expression after CCI at the spinal cord level in both sexes. Relative changes in *ccl2* gene expression normalized to *gapdh* (endogenous control) in SC three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (*$p \leq 0.05$).

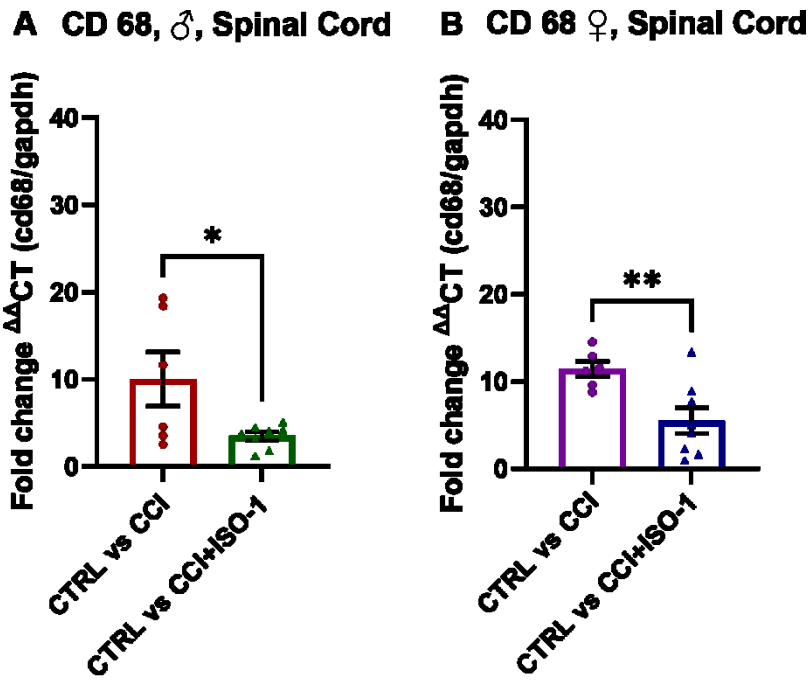

Fig 12.16. ISO-1 effect on CD68 gene expression after CCI at the spinal cord level in both sexes. Relative changes in *cd68* gene expression normalized to *gapdh* (endogenous control) in SC three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (*$p \leq 0.05$, **$p \leq 0.01$).

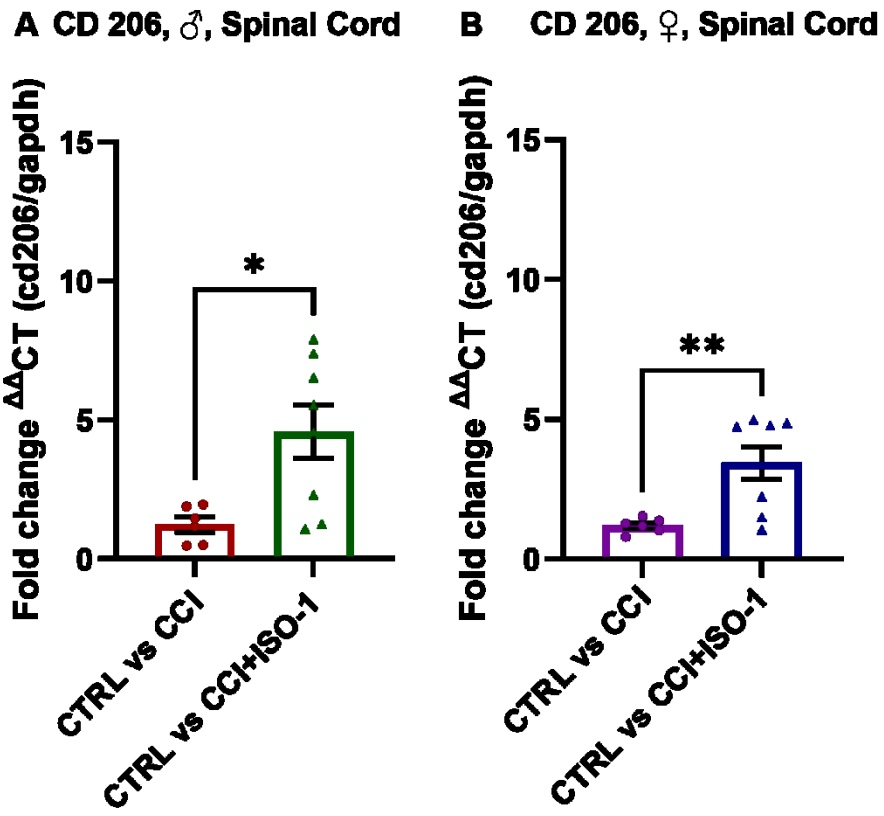

Fig 12.17. ISO-1 effect on CD206 gene expression after CCI at the spinal cord level in both sexes. Relative changes in *cd206* gene expression normalized to *gapdh* (endogenous control) in SC three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (*$p \leq 0.05$, **$p \leq 0.01$).

In males' DRG an increase in RNA level of MIF (CTRL vs CCI: 17.84 ± 2.4, n=5, fig. 12.18), CD74, CCL2 (CTRL vs CCI: 5.9 ± 1.2, n=5, fig. 12.20), CD68 (CTRL vs CCI: 60.2 ± 20.6, n=5, fig. 12.21) and CD206 was shown 3 days after CCI. ISO-1 treatment for three days significantly decreased MIF (CTRL vs CCI+ISO-1: 8.4 ± 1.9, n=8, $p < 0.05$, fig. 12.18), CCL2 (CTRL vs CCI+ISO-1: 2.2 ± 0.6, n=6, $p < 0.05$, fig. 12.20) and CD68 (CTRL vs CCI+ISO-1: 14.1 ± 4.7, n=7, $p < 0.05$, fig. 12.21) RNA expression. ISO-1 did not affect CD74 and CD206 expression.

In females' DRG an increase in RNA level of MIF, CD74 (CTRL vs CCI: 3.1 ± 0.96, n=6, fig. 12.17), CCL2, CD68 (CTRL vs CCI: 67.9 ± 20.9, n=6, fig. 12.19) and CD206 (CTRL vs CCI:

9.1 ± 1.7, n=5, fig.12.20) was shown 3 days after CCI. ISO-1 treatment for three days significantly decreased CD74 (CTRL vs CCI+ISO-1: 1.2 ± 0.2, n=8, $p < 0.05$, fig. 12.17), CD68 (CTRL vs CCI+ISO-1: 14.9 ± 4.5, n=7, $p < 0.05$, fig. 12.19) and CD206 (CTRL vs CCI+ISO-1: 3.3 ± 0.5, n=7, $p < 0.05$, fig. 12.20) RNA expression. MIF inhibitor attenuated CCL2 expression but this effect was not significant. ISO-1 did not affect MIF expression. To conclude, ISO-1 had a different effect on the expression of MIF and CD74 in males and females but it affected CCL2, CD68 and CD206 RNA levels following the same trend in both sexes in DRGs.

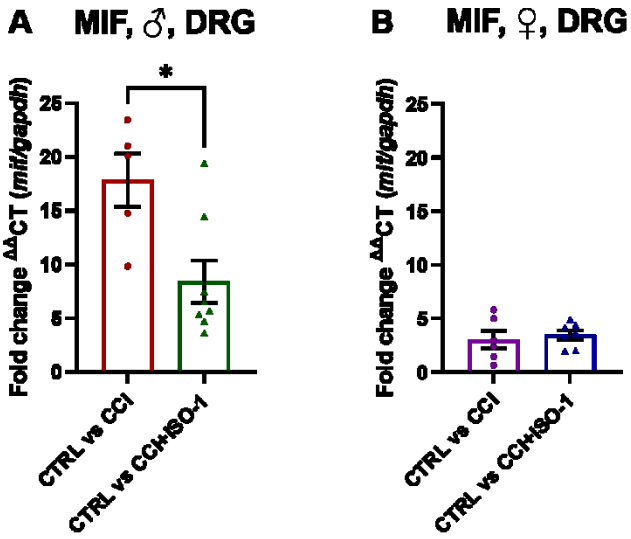

Fig 12.18. ISO-1 effect on MIF gene expression after CCI in DRGs in both sexes. Relative changes in *mif* gene expression normalized to *gapdh* (endogenous control) in DRGs three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (*$p \leq 0.05$).

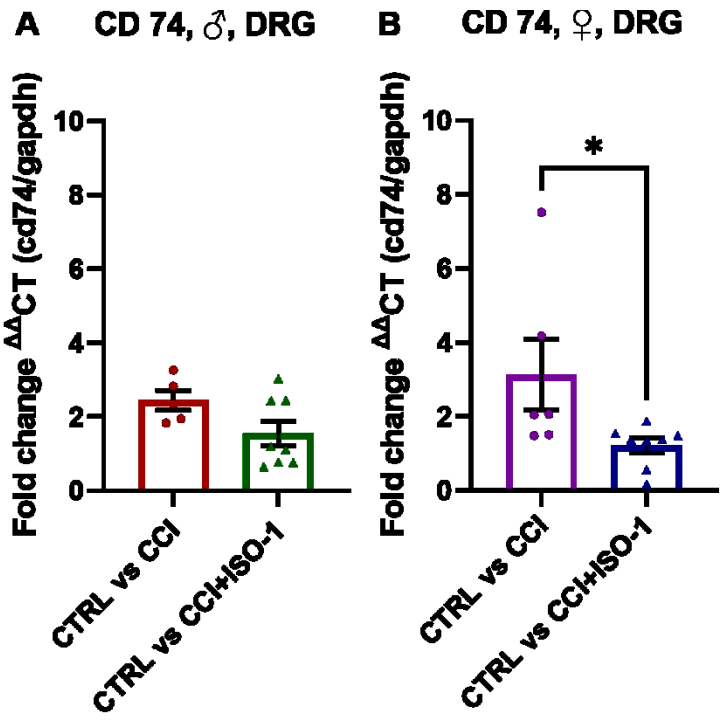

Fig 12.19. ISO-1 effect on CD74 gene expression after CCI in DRGs in both sexes. Relative changes in *cd74* gene expression normalized to *gapdh* (endogenous control) in DRGs three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison.

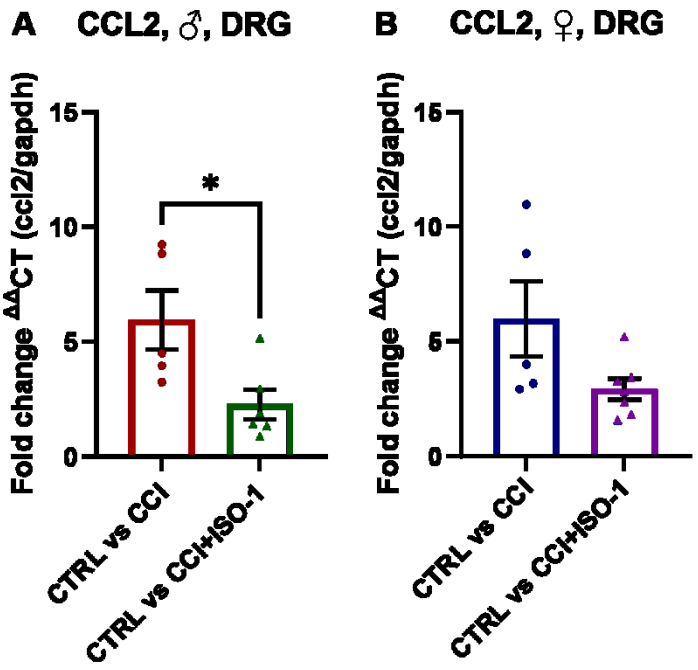

Fig 12.20. ISO-1 effect on CCL2 gene expression after CCI in DRGs in both sexes. Relative changes in *ccl2* gene expression normalized to *gapdh* (endogenous control) in DRGs three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (*$p \leq 0.05$).

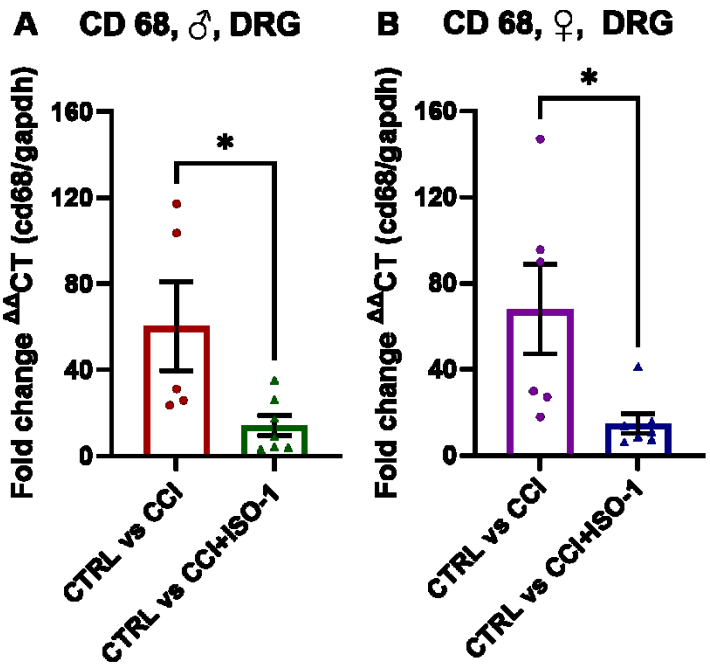

Fig 12.21. ISO-1 effect on CD68 gene expression after CCI in DRGs in both sexes. Relative changes in *cd68* gene expression normalized to *gapdh* (endogenous control) in DRGs three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (*$p \leq 0.05$).

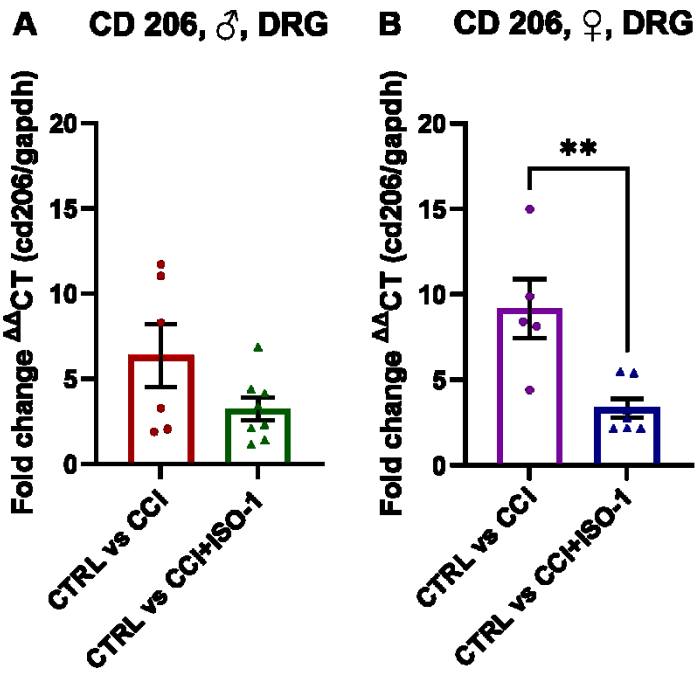

Fig 12.22. ISO-1 effect on CD206 gene expression after CCI in DRGs in both sexes. Relative changes in *cd206* gene expression normalized to *gapdh* (endogenous control) in DRGs three days after CCI and three days after CCI and ISO-1 treatment (20 mg/kg i.p., every day from Day 0 to Day 2) in males (A) and females (B). Data represent Mean ± S.E.M of $2^{-\Delta\Delta}$CT values calculated from six independent experiments (n= 6 animals in each treatment group). Unpaired t test was used for statistical comparison. Asterisks indicate the significance of the comparisons to the CCI group (**$p \leq 0.01$).

12.2 The role of AEA in modulation of nociceptive synaptic transmission under inflammatory condition

This section of the results is dedicated to the modulatory role of AEA at the spinal cord dorsal horn level after induction of peripheral inflammation. The following results constitute a part of the published manuscript Pontearso et al. (Pontearso et al., 2024), which reported the dual effect (inhibitory/excitatory) of AEA on the nociceptive synaptic transmission under control and inflammatory conditions.

Previous studies have shown that at low concentration AEA triggers CB_1 receptors activity, while at a high concentration (higher than 10 uM) AEA activates TRPV1 receptors (Morisset et al., 2001; Ahluwalia et al., 2003). Then, we have studied changes in mEPSC in spinal cord

slices after the application of three increasing concentrations of AEA (1 µM, 10 µM, 30 µM, 4 min each) in naïve conditions. AEA did not affect the mEPSC frequency (control: 100 %, 1 µM AEA: 110.4±11.5 %, 10 µM AEA: 121.1±14.4 %, and 30 µM AEA: 115.6±13.2 %, n=23; Fig. 12.23). Amplitudes of mEPSC did not change during the whole recording (control: 21.4±2.0 pA, 1 µM AEA: 19.3±1.8 pA, 10 µM AEA: 19.1±1.7 pA and 30 µM AEA: 19.1±2.1 pA, n=23). It was noticeable that in the majority of the recorded neurons there was an increase or decrease (>15 %) in the mEPSC frequency in response to increasing concentration of AEA. We sorted the recorded neurons into different populations based on their response to AEA, excitatory or inhibitory effect. In 39.1% of the recorded neurons there was a decrease, while in the 43.5% there was an increase in the frequency of mEPSC and this effect was statistically significant (Fig.12.24).

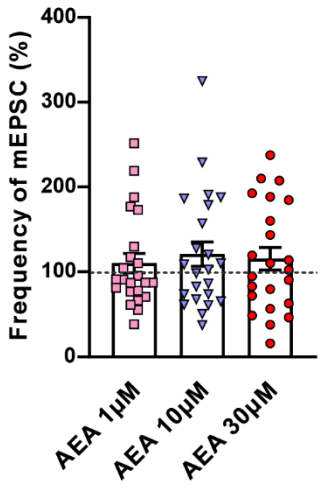

Fig 12.23. In naive conditions, AEA application effect on mEPSC in spinal cord slices. Application of AEA (1 µM, 10 µM, and 30 µM, n=23) did not show any significant effect on mEPSC frequency. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test.

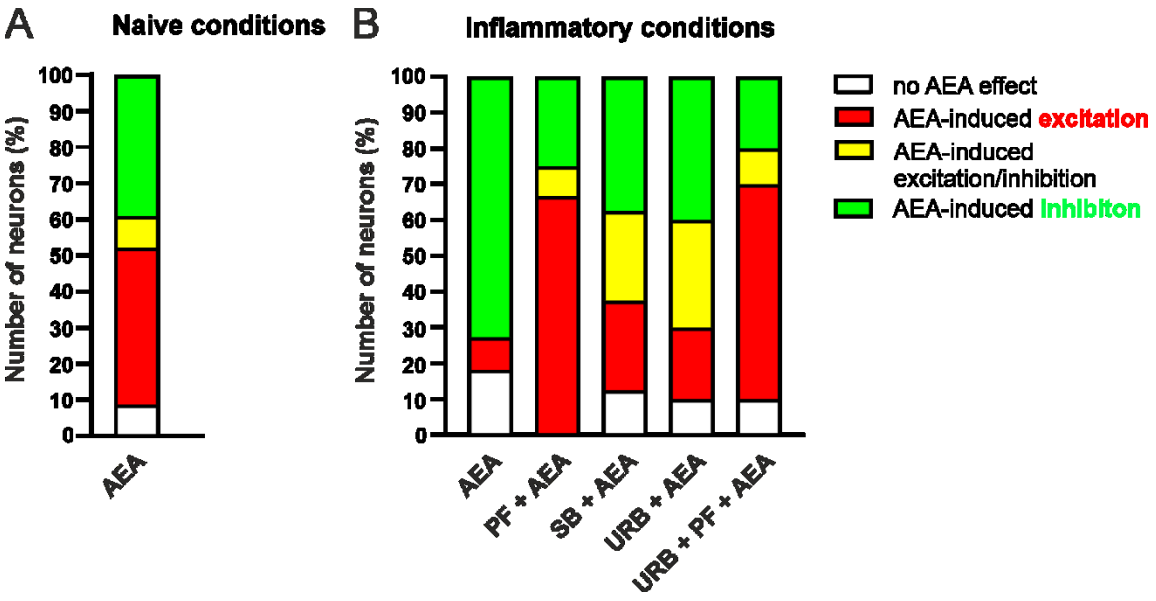

Fig. 12.24. AEA dual effect on synaptic transmission in naïve conditions and after inflammation and inhibition of CB_1, TRPV1, and FAAH (A) The percentage of neurons affected by anandamide (AEA) induced increase or decrease of mEPSC frequency is comparable in naïve conditions. **(B)** The percentage of neurons affected by AEA-induced decrease of mEPSC frequency is higher (73%) compared with neurons affected by AEA-induced increase of mEPSC frequency (9%) in inflammatory conditions. AEA application induced excitation in a higher number of neurons under CB_1 receptor antagonist (PF 514273) effect and under CB_1 receptor antagonist and FAAH (PF 514273/URB 597 co-application) effect.

We have investigated changes in mEPSC in spinal cord slices after the application of AEA and different antagonists/inhibitors 24 hours after subcutaneous carrageenan injection. For the first set of experiments, we applied three increasing concentrations of AEA (1 μM, 10 μM, 30 μM, 4 min each) which reduced the frequency of mEPSC; but, this decrease was not statistically significant on average (Fig. 12.25). We noticed an AEA application-induced inhibitory or excitatory effect and decided to sort neurons based on the responses. The vast majority of neurons 8/11 (72.7 %, Fig. 12.24) showed a significant mEPSC frequency's decrease. Only in 1 (9.1 %) of the 11 neurons AEA evoked an excitatory effect and in 2/11 (18.2 %) recorded neurons (Fig. 12.24) did not affect the mEPSC frequency. The amplitude of mEPSC was not affected by AEA application for the whole recording (control: 20.7±3.0 pA,
1 μM AEA: 18.6±1.4 pA, 10 μM AEA: 18.7±1.0 pA, and 30 μM AEA: 17.2±1.1 pA, n=11).

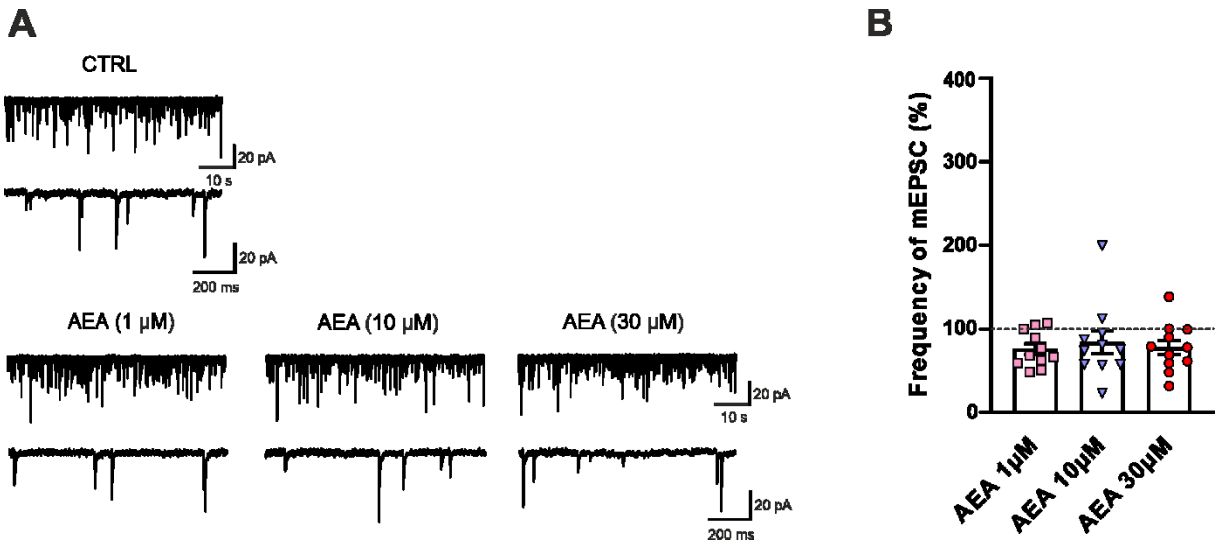

Fig 12.25. Under inflammatory conditions, AEA application effect on mEPSC in spinal cord slices. (A) One minute (top) and one second (bottom) traces of native recordings of mEPSC in control situation and increasing AEA concentration application (1 µM, 10 µM, and 30 µM). (B) Application of AEA (1 µM, 10 µM, and 30 µM, n=11) did not show any significant effect on mEPSC frequency. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test.

12.2.1 The effect of CB_1 receptor inhibition in AEA modulation of nociceptive synaptic transmission under inflammatory condition

Pretreating the slices with PF 514273 (CB_1 receptor antagonist, 0.2 µM, 6 min) did not cause a significant change in frequency of mEPSC. Frequency of mEPSC did not change even when we co-applied AEA (1 µM, 10 µM, 30 µM, 4 min each) and PF 514273 (0.2 µM). Frequency of mEPSC decreased in 3 (25.0 %) and increased significantly in the majority 8 (66.7 %) of the 12 recorded neurons as it is shown in figure 12.24. The mEPSC amplitude did not change during the whole recording (control: 18.5±1.3 pA, PF: 19.4±1.5 pA, 1 µM AEA/PF: 18.3±1.3 pA, 10 µM AEA/PF: 18.5±1.6 pA and 30 µM AEA/PF: 19.1±1.9 pA, n=12). Inhibiting CB_1 receptor under inflammatory condition increased the number of neurons with synaptic input enhanced by the AEA application.

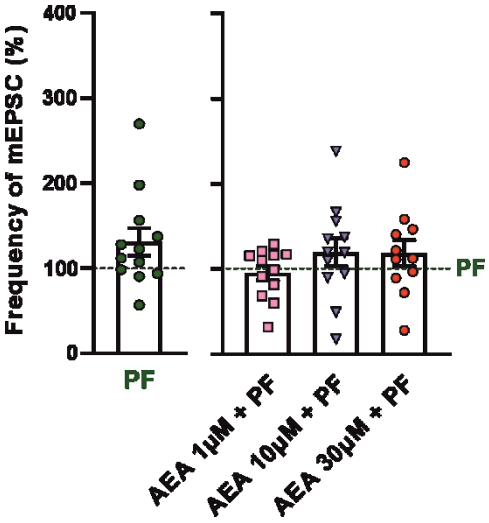

Fig 12.26. Under inflammatory conditions, AEA application effect after CB_1 receptor inhibition on mEPSC in spinal cord slices. PF 514273 (0.2 μM, n=12) application did not elicit a significant change in the mEPSC frequency. Application of AEA (1 μM, 10 μM, and 30 μM) did not significantly change the mEPSC frequency on average when normalized to PF pretreatment. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test.

12.2.2 The role of TRPV1 inhibition on AEA modulation of nociceptive synaptic transmission under inflammatory condition

Application of SB 366791 alone (10 μM, 4 min), a TRPV1 antagonist, caused a decrease in the frequency of mEPSC to 81.4±6.5 % from the control value (n=8, $p<0.05$; Fig. 12.27). Frequency of mEPSC did not change even when we co-applied AEA (1 μM, 10 μM, 30 μM, 4 min each) and SB 366791 (10 μM). Analysing responses in individual neurons highlighted that in 3 (37.5 %) out of 8 neurons there was a decrease in mEPSC frequency and in 2 (25.0 %) out of 8 neurons the mEPSC frequency increased. AEA induced a mixed effect in 2/8 neurons (25.0 %), and only in 1 (12.5 %) out of the 8 neurons recorded the mEPSC frequency did not change (Fig. 12.24). The amplitude of the mEPSC was not affected by the TRPV1 antagonist application (control: 19.0±1.9 pA, SB 366791: 19.2±2.1 pA, 1 μM AEA/SB: 18.7±2.3 pA, 10 μM AEA/SB: 19.2±2.7 pA, 30 μM AEA/SB: 18.2±2.0 pA, n=8). Under inflammatory condition, TRPV1 inhibition reduced the frequency of mEPSC. On the other hand, anandamide did not cause any overall change.

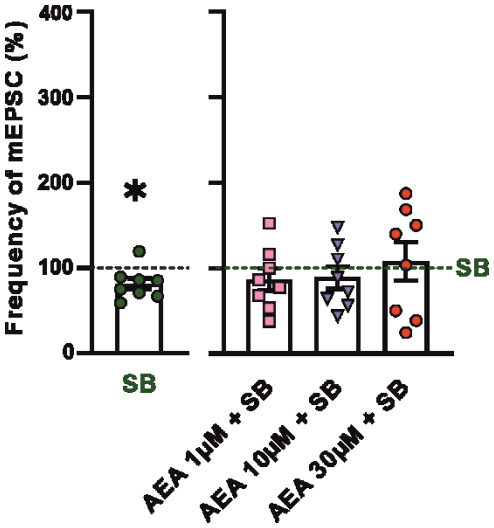

Fig 12.27. Under inflammatory conditions, AEA application effect after TRPV1 inhibition on mEPSC in spinal cord slices. Application of SB 366791 (SB, 10 µM, n=8) TRPV1 antagonist, significantly decreased the mEPSC frequency. Increasing concentrations of AEA did not significantly influence the mEPSC frequency when normalized to SB pretreatment. Statistical analysis: *p<0.05, one-way ANOVA followed by Tukey's multiple comparisons test.

12.2.3 Consequences of FAAH inhibition on AEA modulation of nociceptive synaptic transmission under inflammatory condition

In another set of experiments, we wanted to test the effect of URB 597, inhibitor of AEA degrading enzyme FAAH, on mEPSCs frequency. URB 597 application (1 µM, 6 min) did not affect the mEPSCs frequency. When we applied AEA and URB 597, frequency of mEPSCs were not altered compared to the pretreatment. When we sorted neurons based on the inhibitory or excitatory effect induced by AEA application, we noticed a decrease of mEPSCs frequency in 4 (40.0 %) and increased in 2 (20.0 %) of 10 recorded neurons (fig. 12.24). During all the applications, mEPSCs' amplitudes did not change (control: 18.7±1.6 pA, URB 597: 16.5±1.5 pA, 1 µM AEA/URB: 16.7±1.7 pA, 10 µM AEA/URB: 15.6±1.3 pA and 30 µM AEA/URB: 16.1±1.7 pA, n=10).

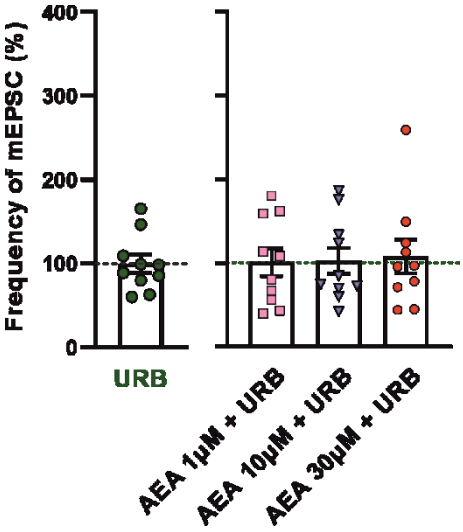

Fig 12.28. Under inflammatory conditions, AEA application effect after FAAH inhibition on mEPSC in spinal cord slices. URB 597 (1 µM, 6 min, n=10) had no significant effect on the mEPSC frequency. AEA increasing concentration did not affect the mEPSC frequency when normalized to URB pretreatment. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test.

12.2.4 The role of co-inhibition of CB_1 and FAAH on AEA modulation of nociceptive synaptic transmission under inflammatory condition

Under inflammatory condition, inhibition of AEA degradation caused by FAAH enzyme did not influence frequency nor amplitude of mEPSCs. AEA induced inhibition or excitation of nociceptive synaptic transmission, but no changes in average. Frequency of mEPSC were not altered by the co-application of URB 597 (1 µM) and PF 514273 (0.2 µM, 6 min), nor when we applied also subsequent concentrations of AEA (1 µM, 10 µM, 30 µM). Sorting of neurons revealed mEPSC frequency decrease only in 4 (20.0 %) and increase significantly in the majority 12 (60.0 %) of 20 neurons (fig.12.24). The amplitude of the mEPSCs did not change for the whole recording under these various applications (control: 16.7±1.0 pA, URB/PF: 15.1±1.2 pA, 1 µM AEA/URB/PF: 14.7±1.1 pA, 10 µM AEA/URB/PF: 14.9±1.1 pA and 30 µM AEA/URB/PF: 14.9±1.1 pA, n=20). Inhibiting pharmacologically FAAH and CB_1 receptors lead to an enhancement of the excitatory synaptic input to the second-order nociceptive neurons caused by the AEA application. A comparison between AEA application with no pretreatment (fig. 12.25) and AEA application with URB and PF514273 pretreatment showed a significant increase of the average mEPSC frequency from all the recorded neurons ($p<0.05$).

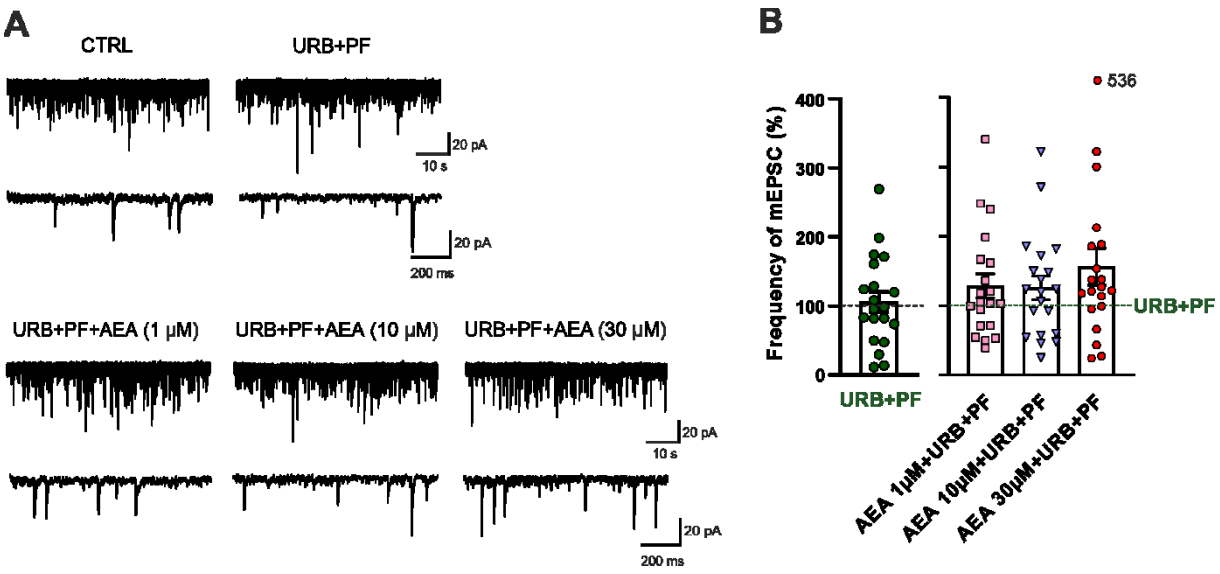

Fig 12.29. Under inflammatory conditions, AEA application effect after CB_1 and FAAH inhibition on mEPSC in spinal cord slices. URB 597 (1 µM) and PF 514273 (0.2 µM, n=20) coapplication did not change the frequency of mEPSC. AEA increased the mEPSC frequency without statistical significance when normalized to URB/PF pretreatment. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test.

12.3 Inflammation-induced mechanical and thermal allodynia did not differ between SGIP1 WT and SGIP1 KO mice

The last part of this work is dedicated to the role of SGIP1 protein in the development of peripheral neuroinflammation. The first set of experiments was focused on the hypersensitivity induced by a single injection of carrageenan on both hindpaws of SGIP1 WT and SGIP1 KO mice. Mice were tested for mechanical and thermal sensitivity before the induction of the inflammation and 24 hours after the carrageenan injection (1%). In both groups, SGIP1 WT and KO, a single injection of carrageenan significantly reduced PWT (in SGIP1 WT, Day 0=7.5 ± 0.3 g, Day 1=2.8 ± 0.2 g, $p < 0.01$, fig 12.28; in SGIP1 KO, Day 0=7.1 ± 0.4 g, Day 1=3.3 ± 0.8 g, $p < 0.01$). For the thermal test, hypersensitivity was well developed in SGIP1 WT and SGIP1 KO (in SGIP1 WT, Day 0=11.1 ± 0.7 s, Day 1=6.7± 0.4 s, $p < 0.05$; in SGIP1 KO, Day 0=11.8 ± 0.4 s, Day 1=6.68± 0.2 s, $p < 0.05$). To conclude, the neuroinflammatory model affected mechanical and thermal sensitivity in WT mice and mice with deletion of SGIP1

protein in a similar extent. These data confirmed that peripheral inflammation was successfully induced because hypersensitivity was well developed and we could use the same mice to prepare acute spinal cord slices and conduct electrophysiology experiments on them.

12.4 WIN 55,212-2-induced decrease of mEPSC frequency was more substantial in SGIP1 KO than in SGIP1 WT mice in a model of peripheral inflammation

For the second set of experiments, we wanted to explore the role of SGIP1 in modulating the nociceptive synaptic transmission at the first synapse of the pain pathway, the spinal cord dorsal horn. Peripheral inflammation was induced by intraplantar carrageenan injection 24h before acute spinal cord slices preparation. CB_1 receptor agonist, WIN 55,212-2, was applied on slices and mEPSC were measured from superficial dorsal horn neurons. WIN 55,212-2 (1uM, 12 minutes) application did not change the mEPSC frequency significantly in SGIP1 WT (n=11) when applied for twelve minutes. Inhibition of mEPSC frequency was significant in SGIP1WT only at the end of the wash-out period (70.95 ± 7.6 %, $p \leq 0.05$, fig 12.30). WIN 55,212-2 (1uM, 12 minutes) application lowered significantly mEPSC frequency in SGIP1 KO (42.3 ± 8.8 %, n=9, $p < 0.001$, fig. 12.30) and this effect persisted during the 10 min wash-out period (35.7 ± 12.6 %, $p < 0.01$). Moreover, when we compared the effect of WIN 55,212-2 between SGIP1 KO and WT, the decrease of mEPSC frequency was higher in SGIP1 KO mice (42.3 ± 8.8 %) compared to (85.9 ± 12.34 %) in WT mice. Under inflammatory conditions, CB_1 receptor agonist application showed more substantial inhibition of nociceptive synaptic transmission in SGIP1 KO than in WT mice.

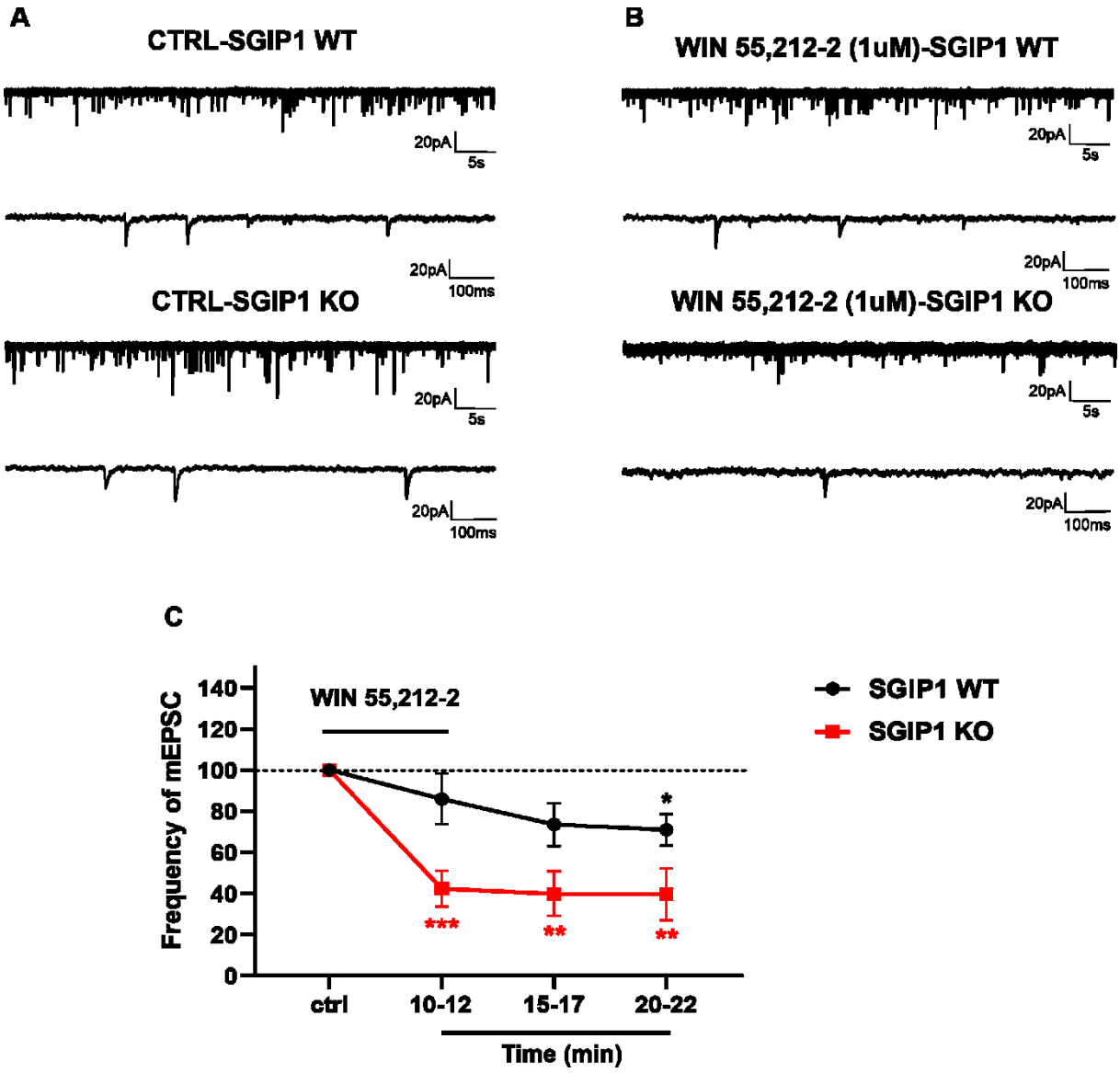

Fig. 12.30. Inhibition of mEPSC by CB_1 receptor agonist was higher in SGIP1 KO than in SGIP1 WT. One minute (top) and one second (bottom) traces of native recordings of mEPSC in control situation **(A)** and inflammation **(B)** from one superficial dorsal horn neuron from SGIP1 WT and SGIP1 KO mice. **(C)** Application of WIN 55,212-2 (1uM, 12 minutes) in slices from SGIP1 WT mice (n=11) decreased the mEPSC frequency significantly at the end of the wash-out ($p \leq 0.05$). Application of WIN 55,212-2 (1uM, 12 minutes) in slices from SGIP1 KO mice (n=9) significantly decreased the mEPSC frequency at 10-12 minutes until the end of the recording. Statistical analysis: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Mixed-effects model followed by Tukey post hoc test.

13 Discussion

Noxious stimuli travels along the axons of nociceptors to reach the first relay station of the pain pathway, the spinal cord dorsal horn. Then, the information is processed by a complex network which includes excitatory, inhibitory interneurons and spinal projection neurons. From the superficial laminas of the spinal cord, nociceptive information is transmitted to the higher brain centres. The focus of this PhD study is on MIF, AEA and SGIP1 protein effects on spinal nociceptive transmission in pathological pain states and the results will be discussed in the next chapters.

13.1 The role of MIF inhibitor, ISO-1, in CCI-induced neuropathic pain

Neuropathic pain arises from an initial injury affecting nervous system tissue which triggers neuroinflammatory mechanisms characterised by involvement of immune cells and release of proinflammatory cytokines. MIF is a proinflammatory cytokine and is costitutively expressed by a variety of immune cell types, including dendritic cells, T and B cells, macrophages, mast cells, eosinophils and neutrophils but also by a variety of tissues like brain, spinal cord and peripheral nervous system (Alexander et al., 2012; Calandra & Roger, 2003; Jankauskas et al., 2019). Moreover, MIF expression, widely present throughout the central and peripheral nervous systems, particularly in sensory transmission regions, significantly increases following nerve injury, reaching a level ~1000-fold higher than other pro-inflammatory cytokines including TNF-a, IL-1β, and IL-6 (X. Wang et al., 2018). It was already demonstrated that, after an inflammatory stimulus, MIF levels increase in microglia, dorsal horn neurons, DRGs and macrophages (Alexander et al., 2012; F. Wang et al., 2011).

Most existing medications for neuropathic pain are characterized by loss of efficacy, tolerance and adverse effects that limit their use; thus, patients require alternative therapeutic options.

This study provides new insights on the importance of the neuro-immune interaction in the modulation of neuropathic pain. Investigating neuro-immune crosstalk has been leading to new discoveries about gender-related differences in the management of neuropathic pain. New

therapies which include targeting of the immune system might lead to an increased quality of life of patients of both sexes affected by neuropathic pain.

In the following chapters, MIF inhibitor effects in vivo and in vitro are discussed.

13.1.1 ISO-1 gender-related regulation of CCI-induced hypersensitivity to mechanical and thermal stimuli in vivo

The function of MIF in modulation of nociception at the peripheral nervous system was previously studied in several pain conditions. It was demonstrated that intraplantar (Alexander et al., 2012) and intrathecal (F. Wang et al., 2011) application of MIF generates pain-related behaviors in naïve animals. On the other hand, in models of sparing nerve injury or chronic constriction injury, MIF inhibition or deletion did not cause hypersensitivity (Alexander et al., 2012) (F. Wang et al., 2011).

In our in vivo experiments, CCI induced mechanical and thermal hyperalgesia from 1 day after injury and persisted until 28 days in mice, male and female. In fact, in the three days in vivo testing, ISO-1 did not change the mechanical hypersensitivity induced by CCI. For this reason, we decided to prolong the time of the experiments and ISO-1 treatment and test the mechanical and thermal hypersensitivity 7 days after the CCI. The timing of the treatment was prolonged as well, from 3 to 7 days. At Day 7, ISO-1 did prevent the mechanical and thermal hyperalgesia in male mice. These results confirm and extend previous studies, which demonstrated an ISO-1 dose-related reduction of mechanical withdrawal threshold and thermal withdrawal latency (F. Wang et al., 2011).

It has become widely accepted that pain processing and the immune system interacts differently between the genders (Mapplebeck et al., 2016; Rosen et al., 2017). Since the inflammation plays a role in nociception and is mediated by different immune cells in males and females, we wanted to explore gender-related differences of the ISO-1 modulation of peripheral neuropathic pain. In previous studies, depleting DRGs macrophages have been used to explore behavioral responses after nerve injury. Removing DRGs macrophages using clodronate attenuated nerve injury-induced mechanical allodynia (Cobos et al., 2018). Shepherd showed that using macrophage Fas-Induced apoptosis (MAFIA) mice in which a genetic deletion leads to macrophages death, mechanical hypersensitivity caused by nerve injury was reduced (Shepherd et al., 2018). It was already shown that depleting macrophages from DRGs reduces signs of neuropathic pain in males and females (Yu et al., 2020). Thus, mechanisms behind

macrophages contribution to modulation of chronic pain differ between sexes. In fact, deleting colony stimulating factor-1 (CSF-1) (a pivotal molecule in microglia and macrophages activity regulation) in a model of peripheral nerve injury impacts negatively macrophages expansion in male but not in female mice (Yu et al., 2020).

In our experiments, we explored the ISO-1 effect on the later stages CCI-induced thermal and mechanical hyperalgesia. So, we extended the time of the experiments to 28 days. ISO-1 treatment was kept the same as in the second set of tests, 7 days long injected once per day. Because of the female bias, our interest was focused on ISO-1 effect on CCI-induced hyperalgesia in both sexes. Tests in male mice show a different duration of ISO-1 effect based on the type of sensitivity. For mechanical sensitivity, ISO-1 analgesic activity lasted for two days after the interruption of the treatment. For thermal sensitivity, ISO-1 antihyperalgesic effect lasted for 7 days once the treatment was stopped. This data suggest that ISO-1 has a prolonged effect on heat rather than on mechanical sensitivity and this might be due to the major role of peripheral sensitization on enhanced thermal but not mechanical responsiveness, which is a main characteristic of central sensitization (Latremoliere & Woolf, 2009). On the other hand, ISO-1 in female mice did not change mechanical neither thermal hypersensitivity. Our results show a sex-related antinociceptive effect of ISO-1. These data might be explained by T-cells related management of pain modulation in females (Sorge et al., 2015). Another study showed that, after deletion of T-cells, inhibiting microglia activity causes an analgesic effect in both sexes. On the other hand, when T-cells are restored only female mice experience pain-like behavior (Sorge et al., 2015). In a neuroinflammatory state, macrophages and T cells collaborate in the nerve infiltration. Macrophages reach the injury site within hours to days from the stimulus, release chemokines and cytokines which stimulate T cells infiltration in the following days (Kim & Moalem-Taylor, 2011). To conclude, ISO-1, through inhibition of MIF tautomerase function, seems to affect macrophages activity but it might not affect T-cells function. For the first time, we showed that intraperitoneal application of ISO-1 cause an analgesic effect that is time and gender related.

13.1.2 Modulation of nociceptive excitatory and inhibitory synaptic transmission by systemic application of MIF inhibitor, ISO-1

A correlation between MIF and nociceptors hyperactivity was already studied in previous works (Bavencoffe et al., 2022; F. Wang et al., 2011). An increase of excitability in small

diameter DRG neurons after MIF acute application was revealed (Alexander et al., 2012). In a following investigation, increasing the concentration of MIF showed a dual, dose-effect dependent action of the pro-inflammatory cytokine in DRGs. Low MIF concentration induced hyperexcitability, meanwhile high MIF concentration induced hypoexcitability (Bavencoffe et al., 2022).

In our experiments, we investigated the effect of systemic application of ISO-1on excitatory and inhibitory synaptic transmission in the lamina I and IIo of the dorsal horn 7 days after CCI and. Previous studies have shown that pathological pain leads to disruption of the balance between excitatory and inhibitory synaptic transmission (Zeilhofer et al., 2012). Therefore, for the first time, the effect of intraperitoneal application of ISO-1 on spinal excitatory and inhibitory post-synaptic currents in a peripheral neuropathic model has been unveiled with this study. Our experiments showed an increase in excitatory synaptic transmission and a decrease in inhibitory synaptic transmission (called disinhibition) 7 days after CCI. This data confirm the loss of the balance between excitatory and inhibitory currents in a peripheral neuropathy pain model.

The anti-nociceptive effect of ISO-1 in our investigation as well as in other scientific works is due to the inhibition of MIF pro-inflammatory activities. MIF, after an inflammatory stimulus, leads to an enhanced release of pro-inflammatory mediators and, as a consequence, to intensification of nociceptive fibers' sensitivity. The result is an increase in the firing of action potentials, when compared with a healthy condition, and augmented pain sensitivity or "hyperalgesia". ISO-1 is one of the small molecule of MIF inhibitors. It binds the tautomerase active site and hinders the cytokine activity (Lubetsky et al., 2002). We showed that 7 days i.p. treatment with MIF inhibitor caused a significant inhibition of the excitatory synaptic transmission indicated by a decreasing in frequencies of sEPSCs and a significant increase of inhibitory synaptic transmission indicated by an increasing in amplitudes of le-IPSCs recorded from excitatory neurons in SCDH. However, sIPSCs frequency did not change in the same experiments. Our results suggest that the imbalance of nociceptive excitatory/inhibitory spinal synaptic transmission mediated by CCI is partially prevented by ISO-1. The discrepancy in ISO-1 effect on spontaneous inhibitory synaptic activity and amplitudes in evoked inhibitory synaptic activity might be due to a preferential influence of ISO-1 on the presynaptic neurons. In a chronic pain state, descending inhibitory pathway function is diminished (Ossipov et al., 2014) and probably ISO-1 was not able to reach higher brain centers and influence descending modulatory circuits activity.

13.1.3 Influence of ISO-1 on macrophages infiltration/proliferation at the site of injury

Scientific works of the last decades have proven that neuropathic pain is precisely regulated by a collaboration between neurons and immune cells which is strictly modulated by molecules of the immune system. After a nerve injury, several immune cells surround the damaged site and regulate neuroinflammation. Macrophages are one of the most important peripheral modulator of neuropathic pain (Ren & Dubner, 2010). The role of macrophages in neuropathic pain was unveiled through previous research. In these investigations, depleting macrophages utilizing a macrophage-targeting toxin or blocking inflammatory cytokines or chemokines generated by macrophages improved neuropathy symptoms (Kobayashi et al., 2015). CD68 is a transmembrane glycoprotein highly expressed by cells in the monocyte lineage, circulating and tissue macrophages. The CD68 expression can be upregulated in inflammatory conditions because of the activation of immune cells upon an appropriate stimulus (Chistiakov et al., 2017). When a nerve is damaged, macrophages are recruited from blood vessels and infiltrate in the site of injury within hours to days from the mechanical insult. In our experiments, we demonstrated that in a neuropathic pain state inhibiting MIF tautomerase activity is essential to reduce macrophages migration to the site of the injury, in this case the sciatic nerve. Blocking MIF function resulted also in a reduction of macrophages infiltration to , the dorsal root ganglia.

Macrophages constitute a significant class of innate immunity cells. Based on the environment surrounding these cells, they can differentiate in subclasses and exert different roles. Two major subtypes of macrophages can be classified, M1 type macrophages, activated by proinflammatory cytokines and conducts bactericidal, proinflammatory and antigen-presenting roles, and M2 macrophages, activated by anti-inflammatory cytokines and carries out an important role in tissue remodelling, allergy and damage repair. Previous investigations revealed that macrophages located in the DRG takes part in the initial phase and persistence of neuropathic pain, and also in the onset and preservation of neuropathic pain-induced mechanical hypersensitivity (Inoue & Tsuda, 2009; Yu et al., 2020). As a consequence, DRGs have been starting to be the target of neuropathic pain treatments (Chen et al., 2022). Our data showed that, after the sciatic nerve injury, total amount of macrophages have increased in the DRG and sciatic nerve, as well as M2 type macrophages. Previous studies have demonstrated that drugs which attenuate neuropathic pain symptoms, induce M2 macrophages proliferation (Popiolek-Barczyk et al., 2015). On the other hand, we saw an increase of M2 macrophages in DRGs and sciatic nerve in a neuropathic pain state and a decrease of M2 macrophages in both tissues after ISO-1 treatment. Explanations to this phenomenon might be the low specificity of

the antibody against the mannose receptor, CD206, and also the expression of this protein not only on M2 type macrophages but also on dendritic cells and monocytes (Azad et al., 2014)

13.1.4 ISO-1 systemic administration affects signs of neuroinflammation

Pain hypersensitivity is one of the main features of peripheral nervous system injury and its onset and persistence is influenced by the neuro-immune crosstalk. In more than one study it was shown that sex differences in pain processing are related with different immune cells involved in the modulation of pain sensitivity. Several investigations have led to the conclusion that female physiology relies on T-cells while male physiology exploits macrophages during the initiation as well as maintenance of the peripheral neuropathy (Sorge et al., 2015a; Vacca et al., 2021). Moreover, in the last decades, the focus of neuropathic pain studies has been moved towards sex differences. In a previous study it was shown that male mice recover faster from CCI compared with female mice (Vacca et al., 2016). In fact, sex differences include also a different timing in the management of pain states, as it was shown by Vacca, in which female mice reacted with an earlier analgesic response than male after the nerve injury but the recovery from the neuropathic pain was incomplete, suggesting that the gender difference starts also from an early stage of the injury (Vacca et al., 2014).

Pain signaling can be enhanced not only by peripheral immune cells but also by immune cells located in the CNS. In fact, in response to an inflammatory stimulus, macrophages as well as spinal microglia lead to pain hypersensitivity. Microglia are considered macrophages of the CNS and after a damage or pathogen stimulation, they can quickly change gene and/or protein surface expression and their morphology.

In our molecular biology experiments, we evaluated ISO-1 activity on genes expression related with MIF and neuroinflammation in a early stage of peripheral neuropathic pain model. A difference was shown in MIF-CD74 pathway between males and females, while expression of CCL2, CD68 and CD206 followed similar trend in both spinal cord and DRG tissues. In fact, CD68 mRNA level was decreased after ISO-1 treatment in both genders in spinal cord and DRGs. CCL2 mRNA levels were also attenuated in spinal cord and DRGs but the effect was stronger in male mice. CCL2 is a cytokine which function is strictly related with macrophages recruitment (Kanda et al., 2006), so the upregulation in a CCI model and downregulation after ISO-1 treatment is due to the inflammation process typical of the neuropathy which is contrasted by systemical application of MIF inhibitor. On the other hand, ISO-1 on CD206 mRNA level induced a dual effect, leading to an increase of CD206 expression in spinal cord

and decrease in CD206 expression in DRGs in both sexes. The tissue-related difference might be due to a faster effect of ISO-1 in the periphery because of the systemical application,different cell population and different regulatory mechanisms in spinal cord and DRGs. For what concern the discrepancy in MIF and CD74 expression, it might be due to the different immune cells type, timing and involvement in modulation of peripheral neuropathy in males and females (Vacca et al., 2014). In fact, ISO-1 did not affect MIF and CD74 expression in females in spinal cord. In females DRGs, CD74 level decreased. In males, it appears that ISO-1 attenuated MIF expression in spinal cord and DRGs but it did not affect CD74 expression in DRGs.

13.2 The Endocannabinoid AEA effect on synaptic transmission at the spinal cord level

Numerous papers have shown that AEA plays a pivotal role in pain modulation through the binding with two receptors, CB_1 and TRPV1. The affinity of AEA for these two receptors may leads to a dual role of the endogenous cannabinoid in the modulation of nociceptive pain at the spinal cord level.

In this work, AEA effect on excitatory synaptic transmission in spinal cord slices in an inflammatory state was studied. AEA application differently affected the neurotransmitter release from primary afferent fibers measured by mEPSC frequency change.

13.3 The role of CB_1, TRPV1 and FAAH inhibition in AEA-mediated effecton excitatory postsynaptic currents

In our previous studies (Nerandzic et al., 2018; Spicarova et al., 2023)we demonstrated a different modulatory mechanisms underlying AEA mediated inhibition of synaptic transmission in naïve and inflammatory conditions. Data from this study indicated that AEA precursor 20:4-NAPE caused an inhibitory effect in naïve animals mediated by CB_1 receptor while TRPV1 receptor activity was involved in an inflammatory state. To show that AEA was synthetized from 20:4-NAPE, we applied anandamide-synthesizing enzyme N-acyl

phosphatidylethanolamine phospholipase D (NAPE-PLD) inhibitor LEI-401 on the slices before 20:4-NAPE application. As a result, the inhibitory effect was blocked.

To continue with our investigation, we investigated the effect of external AEA application on spinal cord slices. In naïve conditions there was a balance in dorsal horn neurons targeted by AEA-inhibition and –excitation of synaptic transmission from primary afferent neurons. After peripheral inflammation, AEA-mediated mainly the inhibition of glutamate release from primary nociceptive fibers. In fact, a more detailed analysis highlighted that mEPSC frequency modulation by AEA application was mainly inhibitory in most of the superficial dorsal horn neurons (73%). Our data are in accordance with a previous work that demonstrated spinal AEA administration has no effect on neuronal responses evoked by transcutaneous electrical stimulation of primary afferent fibers in a control situation. On the other hand, a reduction of these responses was mediated by AEA via CB_1 receptor activation after inflammation (Harris et al., 2000).

A potentiation of synaptic input induced by AEA was shown after blocking CB_1 receptor and enhanced by co-inhibition of CB_1/FAAH. We noticed that AEA-induced excitation in individual neurons was stronger after co-inhibition of CB_1/FAAH compared with blocking of CB_1 receptor. During CB_1/FAAH inhibition, mEPSC frequency increase was caused by the lowest AEA concentration (1 μM) application also. TRPV1 channel involvement, when CB_1 receptor antagonist is applied, in excitation of spontaneous EPSC was also elucidated in our earlier experiments in which 20:4-NAPE, AEA precursor, was used instead of direct application AEA (Nerandzic et al., 2018). This data supports the hypothesis that, in inflammatory conditions, AEA-mediated inhibitory effect is mainly regulated by CB_1 receptor activity, which prevented the excitation of synaptic transmission modulated by AEA activation of TRPV1 and blocking of AEA degradation (Luo et al., 2002).

We noticed strong differences in the AEA modulation of nociceptive transmission when AEA is synthetized from 20:4-NAPE via NAPE-PLD compared with exogenous addition of AEA.

To conclude, the endocannabinoid/endovanilloid system is characterized by various changes in inflammatory conditions and synthesis of AEA from the precursor by local enzymes may be advantageous for analgesia than external AEA administration.

13.4 SGIP1 protein influence on nociception in inflammatory conditions

Several scientific works have demonstrated that cannabinoids are involved in suppression of behavioural reactions to noxious stimuli and the analgesic effects of cannabinoids are modulated also at the spinal cord level. Cannabinoids have been administrated spinally and their antinociceptive effect was shown by behavioural (Kosersky et al., 1973; Tsou et al., 1996; Richardson et al., 1998), electrophysiological (Drew et al., 2000; Harris et al., 2000; Chapman, 2001) and neurochemical studies (Tsou et al., 1996; Hohmann & Herkenham, 1999; Richardson et al., 1998a). Δ^9-THC increased PWT after inflammation of the hindpaw induced by carrageenan injection (Kosersky et al., 1973). In another study, after intradermal capsaicin injection, systemic application of WIN55,212-2 reduced mechanical and thermal hypersensitivity via CB_1 receptor action (Martin et al., 1996).

Endocannabinoids are synthetized into the postsynaptic neuron, they are secreted into the synapse and operate at the presynaptic level in a retrograde way to induce the secretion of various neurotransmitters. CB_1 is a G protein coupled receptor and the binding with its ligand cause an inhibition of Ca2+ influx mediated by voltage-gated Ca2+ channles. The consequence is an hyperpolarization of the presynaptic element and modulation of neurotransmitter release, such as decrease in glutamate release, and synaptic transmission (Barrie N 2017).

Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting protein 1 (SGIP1) is present at the PNS and CNS, specifically at the presynaptic area (Wilhelm et al., 2014). SGIP1 modulates the clatrin mediated endocytosis (CME) of CB_1 receptor (Gazdarica et al., 2022). Moreover, interaction between arrestin beta and CB_1R is stimulated by SGIP1 and is weaker when SGIP1 gene is deleted (Hájková et al., 2016). Thus, SGIP1 interferes with CB_1 internalization and desensitization, causing a lower affinity of the CB_1 for its ligand. In presence of SGIP1, CB_1 receptor and arrestin interact for a longer time (Hájková et al., 2016)

The electrophysiology results showed differences between the two groups in spinal cord slices taken from the same mice. When we were recording mEPSC from SGIP1 WT and KO superficial dorsal horn neurons, we applied CB_1 receptor agonist, WIN55,212-2, and we noticed a stronger inhibition of excitatory synaptic transmission when SGIP1 protein was deleted. Our electrophysiological experiments indicate that SGIP1 interaction with CB_1R on primary afferent terminals in the spinal cord dorsal horn could play an important role in nociceptive transmission, especially under inflammatory conditions. After peripheral inflammation,

avoiding the SGIP1 interaction with presynaptic CB_1 receptors increased the antinociceptive effect of CB_1 receptor stimulation. In this process, the desensitization/internalization of CB_1 receptors may have a role (Hájková et al., 2016).

14 Conclusions

Pain is a physiological response of our body to an endogenous or exogenous stimulus which is perceived as a threat. The switching from physiological to pathological pain happens when the stimulus is too intense and too prolonged and it might lead to chronic pain.

In superficial dorsal horn neurons, regulation of synaptic transmission plays a pivotal role in nociceptive signaling. When a nervous structure is damaged, inflammatory reactions begin and many regulatory mechanisms are involeved in the management of neuroinflammation in the periphery, DRG, but also central sites, spinal cord dorsal horn. The use of anti-inflammatory drugs to treat neuroinflammation is widely proven as an effective therapy (Janjic et al., 2018; Deal et al., 2022). Therefore, the first part of this PhD thesis was focused on testing the effect of an anti-inflammatory substance ISO-1, a MIF inhibitor, as drug for neuroinflammatory issues associated with neuropathic pain. Secondly, the cannabinoid system was investigated in order to treat neuroinflammation due to a main feature of the endocannabinoids, which is the inhibition of glutamate release into the synaptic cleft and consequent analgesic effect.

Our results showed that ISO-1 inhibition of MIF tautomerase active site induces complex reactions in vivo and in vitro which are sex-dependent as well. The analgesic effect of ISO-1 treatment in vivo was time and sex dependent. It was shown before that MIF inhibitor decreases sensitivity to the mechanical stimulus in a neuropathic pain condition (F. Wang et al., 2011). Our results thus suggest that ISO-1 affects hypersensitivity in a different extent based on the stimulus and its effect might be exerted on macrophages which are not the first cells involved in females immune reaction. Electrophysiology data showed how ISO-1 impacted sEPSC frequency and le-IPSC amplitudes, restoring the loss of balance between excitatory and inhibitory transmission. Moreover, ISO-1 significantly reduced macrophages infiltration in two distinct compartments, DRGs and sciatic nerve, and this is in accordance with the effect showed in behavioural experiments even though data about anti-inflammatory macrophages need further investigations. On a gene expression level, our findings confirm ISO-1 activity targeted

on macrophages and they might suggest a different regulation of ISO-1 on T-cells since MIF and CD74 did not change their expression profile after the treatment in spinal cord and DRG in females, which rely on adaptive immune system modulation of the inflammation (Sorge et al., 2015a; Vacca et al., 2021). On the other hand, CD68 and CD206 gene expression in males and females follow the same regulation 3 days after ISO-1 treatment. In the end, targeting MIF activity might be a good starting point for new cytokine-related therapies of peripheral neuroinflammatory diseases. On the other hand, these results suggest a different approach for neuropathic pain treatment in females and support data from previous works.

AEA modulation of nociceptive synaptic transmission is shifted towards an AEA-mediated inhibition in a model of peripheral inflammation induced by carrageenan. Inhibiting CB_1 and FAAH together under inflammatory conditions increment the amount of superficial dorsal horn neurons in which the excitatory synaptic input was increased starting from the lowest concentration of AEA. Excitatory synaptic transmission modulation at the spinal cord level by AEA may highlight the analgesic effect of AEA (Pontearso et al., 2024). However, modulating the levels of endogenously synthesized AEA using its precursor may be preferential for inducing anti-nociception (Spicarova et al., 2023)

The experimental work about SGIP1 protein showed that only after application of the CB_1 agonist it is possible to highlight the importance of SGIP1 in the desensitization of CB_1 receptors. In fact, WIN 55,212-2 exerts a stronger inhibitory effect in an inflammatory model only when SGIP1 protein is absent, suggesting that targeting this protein might be a good potential target for neuroinflammation treatment.

In the end, the findings characterising this doctoral work reveal novel potential processes of neuroinflammatory pain state development, highlight MIF as an important target for the potential development of improved cytokine-target treatment, and confirm the crucial role of neuroimmune crosstalk in neuroinflammatory pain treatment. Additionally, this work also highlight another key system to take into consideration as potential therapeutic target for the neuroinflammation, which is the endocannabinoid system. the results presented will be useful as a basis for following research and new treatments for neuropathic and neuroinflammatory pain, helping patients affected by this disease.

15 REFERENCES

Adamek, P., Heles, M., Bhattacharyya, A., Pontearso, M., Slepicka, J., & Palecek, J. (2022). Dual PI3Kδ/γ Inhibitor Duvelisib Prevents Development of Neuropathic Pain in Model of Paclitaxel-Induced Peripheral Neuropathy. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *42*(9), 1864–1881. https://doi.org/10.1523/JNEUROSCI.1324-21.2021

Ahluwalia, J., Urban, L., Bevan, S., & Nagy, I. (2003). Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro. *The European Journal of Neuroscience*, *17*(12), 2611–2618. https://doi.org/10.1046/j.1460-9568.2003.02703.x

Alexander, J. K., Cox, G. M., Tian, J.-B., Zha, A. M., Wei, P., Kigerl, K. A., Reddy, M. K., Dagia, N. M., Sielecki, T., Zhu, M. X., Satoskar, A. R., McTigue, D. M., Whitacre, C. C., & Popovich, P. G. (2012). Macrophage migration inhibitory factor (MIF) is essential for inflammatory and neuropathic pain and enhances pain in response to stress. *Experimental Neurology*, *236*(2), 351–362. https://doi.org/10.1016/j.expneurol.2012.04.018

Armstrong, S. A., & Herr, M. J. (2024). Physiology, Nociception. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK551562/

Atwood, B. K., & Mackie, K. (2010). CB2: A cannabinoid receptor with an identity crisis. *British Journal of Pharmacology*, *160*(3), 467–479. https://doi.org/10.1111/j.1476-5381.2010.00729.x

Austin, P. J., & Moalem-Taylor, G. (2010). The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. *Journal of Neuroimmunology*, *229*(1–2), 26–50. https://doi.org/10.1016/j.jneuroim.2010.08.013

Azad, A. K., Rajaram, M. V. S., & Schlesinger, L. S. (2014). Exploitation of the Macrophage Mannose Receptor (CD206) in Infectious Disease Diagnostics and Therapeutics. *Journal of Cytology & Molecular Biology*, *1*(1), 1000003. https://doi.org/10.13188/2325-4653.1000003

Bacher, M., Meinhardt, A., Lan, H. Y., Dhabhar, F. S., Mu, W., Metz, C. N., Chesney, J. A., Gemsa, D., Donnelly, T., Atkins, R. C., & Bucala, R. (1998). MIF expression in the rat brain: Implications for neuronal function. *Molecular Medicine*, *4*(4), 217–230.

Bannister, K., & Dickenson, A. H. (2016). What do monoamines do in pain modulation? *Current Opinion in Supportive and Palliative Care*, *10*(2), 143–148. https://doi.org/10.1097/SPC.0000000000000207

Barabas, M. E., Mattson, E. C., Aboualizadeh, E., Hirschmugl, C. J., & Stucky, C. L. (2014). Chemical structure and morphology of dorsal root ganglion neurons from naive and inflamed mice. *The Journal of Biological Chemistry*, *289*(49), 34241–34249. https://doi.org/10.1074/jbc.M114.570101

Barclay, J., Clark, A. K., Ganju, P., Gentry, C., Patel, S., Wotherspoon, G., Buxton, F., Song, C., Ullah, J., Winter, J., Fox, A., Bevan, S., & Malcangio, M. (2007). Role of the cysteine

protease cathepsin S in neuropathic hyperalgesia. *Pain*, *130*(3), 225–234. https://doi.org/10.1016/j.pain.2006.11.017

Barrette, B., Hébert, M.-A., Filali, M., Lafortune, K., Vallières, N., Gowing, G., Julien, J.-P., & Lacroix, S. (2008). Requirement of myeloid cells for axon regeneration. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *28*(38), 9363–9376. https://doi.org/10.1523/JNEUROSCI.1447-08.2008

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell*, *139*(2), 267–284. https://doi.org/10.1016/j.cell.2009.09.028

Basbaum, A. I., Gautron, M., Jazat, F., Mayes, M., & Guilbaud, G. (1991). The spectrum of fiber loss in a model of neuropathic pain in the rat: An electron microscopic study. *Pain*, *47*(3), 359–367. https://doi.org/10.1016/0304-3959(91)90229-Q

Bavencoffe, A., Spence, E. A., Zhu, M. Y., Garza-Carbajal, A., Chu, K. E., Bloom, O. E., Dessauer, C. W., & Walters, E. T. (2022). Macrophage Migration Inhibitory Factor (MIF) Makes Complex Contributions to Pain-Related Hyperactivity of Nociceptors after Spinal Cord Injury. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *42*(27), 5463–5480. https://doi.org/10.1523/JNEUROSCI.1133-21.2022

Bendszus, M., & Stoll, G. (2003). Caught in the act: In vivo mapping of macrophage infiltration in nerve injury by magnetic resonance imaging. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *23*(34), 10892–10896. https://doi.org/10.1523/JNEUROSCI.23-34-10892.2003

Bennett, G. J., & Xie, Y.-K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*, *33*(1), 87–107. https://doi.org/10.1016/0304-3959(88)90209-6

Biedermann, T., Kneilling, M., Mailhammer, R., Maier, K., Sander, C. A., Kollias, G., Kunkel, S. L., Hültner, L., & Röcken, M. (2000). Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *The Journal of Experimental Medicine*, *192*(10), 1441–1452. https://doi.org/10.1084/jem.192.10.1441

Bloom, B. R., & Bennett, B. (1966). Mechanism of a Reaction in Vitro Associated with Delayed-Type Hypersensitivity. *Science*, *153*(3731), 80–82. https://doi.org/10.1126/science.153.3731.80

Bourne, S., Machado, A. G., & Nagel, S. J. (2014). Basic anatomy and physiology of pain pathways. *Neurosurgery Clinics of North America*, *25*(4), 629–638. https://doi.org/10.1016/j.nec.2014.06.001

Braz, J., Solorzano, C., Wang, X., & Basbaum, A. I. (2014). Transmitting pain and itch messages: A contemporary view of the spinal cord circuits that generate Gate Control. *Neuron*, *82*(3), 522–536. https://doi.org/10.1016/j.neuron.2014.01.018

Browne, T. J., Smith, K. M., Gradwell, M. A., Iredale, J. A., Dayas, C. V., Callister, R. J., Hughes, D. I., & Graham, B. A. (2021). Spinoparabrachial projection neurons form distinct classes in the mouse dorsal horn. *Pain*, *162*(7), 1977–1994. https://doi.org/10.1097/j.pain.0000000000002194

Brück, W. (1997). The role of macrophages in Wallerian degeneration. *Brain Pathology (Zurich, Switzerland)*, *7*(2), 741–752. https://doi.org/10.1111/j.1750-3639.1997.tb01060.x

Butt, A. M., & Bay, V. (2011). Axon-glial interactions in the central nervous system. *Journal of Anatomy*, *219*(1), 1–1. https://doi.org/10.1111/j.1469-7580.2011.01401.x

Cabral, G. A., Ferreira, G. A., & Jamerson, M. J. (2015). Endocannabinoids and the Immune System in Health and Disease. *Handbook of Experimental Pharmacology*, *231*, 185–211. https://doi.org/10.1007/978-3-319-20825-1_6

Calandra, T., Bernhagen, J., Mitchell, R. A., & Bucala, R. (1994). The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *The Journal of Experimental Medicine*, *179*(6), 1895–1902. https://doi.org/10.1084/jem.179.6.1895

Calandra, T., & Roger, T. (2003). Macrophage migration inhibitory factor: A regulator of innate immunity. *Nature Reviews. Immunology*, *3*(10), 791–800. https://doi.org/10.1038/nri1200

Cao, L., & DeLeo, J. A. (2008). CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *European Journal of Immunology*, *38*(2), 448–458. https://doi.org/10.1002/eji.200737485

Chalimoniuk, M., King-Pospisil, K., Metz, C. N., & Toborek, M. (2006). Macrophage migration inhibitory factor induces cell death and decreases neuronal nitric oxide expression in spinal cord neurons. *Neuroscience*, *139*, 1117–1128. https://doi.org/10.1016/j.neuroscience.2005.12.056

Chapman, V. (2001). Functional changes in the inhibitory effect of spinal cannabinoid (CB) receptor activation in nerve injured rats. *Neuropharmacology*, *41*(7), 870–877. https://doi.org/10.1016/s0028-3908(01)00125-3

Chen, H., Jiang, L., Zhang, D., Chen, J., Luo, X., Xie, Y., Han, T., Wang, L., Zhang, Z., Zhou, X., & Yan, H. (2022). Exploring the Correlation Between the Regulation of Macrophages by Regulatory T Cells and Peripheral Neuropathic Pain. *Frontiers in Neuroscience*, *16*, 813751. https://doi.org/10.3389/fnins.2022.813751

Cheng, T., Xu, Z., & Ma, X. (2022). The role of astrocytes in neuropathic pain. *Frontiers in Molecular Neuroscience*, *15*. https://doi.org/10.3389/fnmol.2022.1007889

Chistiakov, D. A., Killingsworth, M. C., Myasoedova, V. A., Orekhov, A. N., & Bobryshev, Y. V. (2017). CD68/macrosialin: Not just a histochemical marker. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *97*(1), 4–13. https://doi.org/10.1038/labinvest.2016.116

Clark, A. K., Gentry, C., Bradbury, E. J., McMahon, S. B., & Malcangio, M. (2007). Role of spinal microglia in rat models of peripheral nerve injury and inflammation. *European Journal of Pain (London, England)*, *11*(2), 223–230. https://doi.org/10.1016/j.ejpain.2006.02.003

Clatworthy, A. L., Illich, P. A., Castro, G. A., & Walters, E. T. (1995). Role of peri-axonal inflammation in the development of thermal hyperalgesia and guarding behavior in a rat model of neuropathic pain. *Neuroscience Letters*, *184*(1), 5–8. https://doi.org/10.1016/0304-3940(94)11154-b

Cobos, E. J., Nickerson, C. A., Gao, F., Chandran, V., Bravo-Caparrós, I., González-Cano, R., Riva, P., Andrews, N. A., Latremoliere, A., Seehus, C. R., Perazzoli, G., Nieto, F. R., Joller, N., Painter, M. W., Ma, C. H. E., Omura, T., Chesler, E. J., Geschwind, D. H., Coppola, G., … Costigan, M. (2018). Mechanistic Differences in Neuropathic Pain Modalities Revealed by Correlating Behavior with Global Expression Profiling. *Cell reports*, *22*(5), 1301–1312. https://doi.org/10.1016/j.celrep.2018.01.006

Coleman, M. (2005). Axon degeneration mechanisms: Commonality amid diversity. *Nature Reviews. Neuroscience*, *6*(11), 889–898. https://doi.org/10.1038/nrn1788

Colonna, M., & Butovsky, O. (2017). Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annual review of immunology*, *35*, 441–468. https://doi.org/10.1146/annurev-immunol-051116-052358

Costigan, M., Moss, A., Latremoliere, A., Johnston, C., Verma-Gandhu, M., Herbert, T. A., Barrett, L., Brenner, G. J., Vardeh, D., Woolf, C. J., & Fitzgerald, M. (2009). T-Cell Infiltration and Signaling in the Adult Dorsal Spinal Cord Is a Major Contributor to Neuropathic Pain-Like Hypersensitivity. *The Journal of Neuroscience*, *29*(46), 14415–14422. https://doi.org/10.1523/JNEUROSCI.4569-09.2009

Costigan, M., Scholz, J., & Woolf, C. J. (2009). Neuropathic pain: A maladaptive response of the nervous system to damage. *Annual Review of Neuroscience*, *32*, 1–32. https://doi.org/10.1146/annurev.neuro.051508.135531

David, J. R. (1966). Delayed hypersensitivity in vitro: Its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proceedings of the National Academy of Sciences of the United States of America*, *56*(1), 72–77.

Davies, A. L., Hayes, K. C., & Dekaban, G. A. (2007). Clinical correlates of elevated serum concentrations of cytokines and autoantibodies in patients with spinal cord injury. *Archives of Physical Medicine and Rehabilitation*, *88*(11), 1384–1393. https://doi.org/10.1016/j.apmr.2007.08.004

Deal, B., Reynolds, L. M., Patterson, C., Janjic, J. M., & Pollock, J. A. (2022). Behavioral and inflammatory sex differences revealed by celecoxib nanotherapeutic treatment of peripheral neuroinflammation. *Scientific Reports*, *12*(1), 8472. https://doi.org/10.1038/s41598-022-12248-8

Dietrich, A., & McDaniel, W. F. (2004). Endocannabinoids and exercise. *British Journal of Sports Medicine*, *38*(5), 536–541. https://doi.org/10.1136/bjsm.2004.011718

Drew, L. J., Harris, J., Millns, P. J., Kendall, D. A., & Chapman, V. (2000). Activation of spinal cannabinoid 1 receptors inhibits C-fibre driven hyperexcitable neuronal responses and increases [35S]GTPgammaS binding in the dorsal horn of the spinal cord of noninflamed and inflamed rats. *The European Journal of Neuroscience*, *12*(6), 2079–2086. https://doi.org/10.1046/j.1460-9568.2000.00101.x

Dubový, P. (2011). Wallerian degeneration and peripheral nerve conditions for both axonal regeneration and neuropathic pain induction. *Annals of Anatomy = Anatomischer Anzeiger: Official Organ of the Anatomische Gesellschaft*, *193*(4), 267–275. https://doi.org/10.1016/j.aanat.2011.02.011

Fehrenbacher, J. C., Vasko, M. R., & Duarte, D. B. (2012). Models of Inflammation: Carrageenan- or Complete Freund's Adjuvant (CFA)–Induced Edema and Hypersensitivity in the Rat. *Current Protocols in Pharmacology*, *56*(1), 5.4.1-5.4.4. https://doi.org/10.1002/0471141755.ph0504s56

Felder, C. C., Joyce, K. E., Briley, E. M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A. L., & Mitchell, R. L. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Molecular Pharmacology*, *48*(3), 443–450.

Fernandez-Zafra, T., Gao, T., Jurczak, A., Sandor, K., Hore, Z., Agalave, N. M., Su, J., Estelius, J., Lampa, J., Hokfelt, T., Wiesenfeld-Hallin, Z., Xu, X., Denk, F., & Svensson, C. I. (2019). Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis. *Pain*, *160*(1), 224–236. https://doi.org/10.1097/j.pain.0000000000001394

Frias, B., & Merighi, A. (2016). Capsaicin, Nociception and Pain. *Molecules (Basel, Switzerland)*, *21*(6), 797. https://doi.org/10.3390/molecules21060797

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., & Casellas, P. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *European Journal of Biochemistry*, *232*(1), 54–61. https://doi.org/10.1111/j.1432-1033.1995.tb20780.x

Galli, S. J., Kalesnikoff, J., Grimbaldeston, M. A., Piliponsky, A. M., Williams, C. M. M., & Tsai, M. (2005). Mast cells as «tunable» effector and immunoregulatory cells: Recent advances. *Annual Review of Immunology*, *23*, 749–786. https://doi.org/10.1146/annurev.immunol.21.120601.141025

Gaudet, A. D., Popovich, P. G., & Ramer, M. S. (2011). Wallerian degeneration: Gaining perspective on inflammatory events after peripheral nerve injury. *Journal of Neuroinflammation*, *8*(1), 110. https://doi.org/10.1186/1742-2094-8-110

Gazdarica, M., Noda, J., Durydivka, O., Novosadova, V., Mackie, K., Pin, J.-P., Prezeau, L., & Blahos, J. (2022). SGIP1 modulates kinetics and interactions of the cannabinoid receptor 1 and G protein-coupled receptor kinase 3 signalosome. *Journal of Neurochemistry*, *160*(6), 625–642. https://doi.org/10.1111/jnc.15569

Gilligan, J. P., Lovato, S. J., Erion, M. D., & Jeng, A. Y. (1994). Modulation of carrageenan-induced hind paw edema by substance P. *Inflammation*, *18*(3), 285–292. https://doi.org/10.1007/BF01534269

Giuffre, B. A., Black, A. C., & Jeanmonod, R. (2024). Anatomy, Sciatic Nerve. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK482431/

Glass, J. D., Culver, D. G., Levey, A. I., & Nash, N. R. (2002). Very early activation of m-calpain in peripheral nerve during Wallerian degeneration. *Journal of the Neurological Sciences*, *196*(1–2), 9–20. https://doi.org/10.1016/s0022-510x(02)00013-8

Grieb, G., Merk, M., Bernhagen, J., & Bucala, R. (2010). Macrophage migration inhibitory factor (MIF): A promising biomarker. *Drug News & Perspectives*, *23*(4), 257–264. https://doi.org/10.1358/dnp.2010.23.4.1453629

Hájková, A., Techlovská, Š., Dvořáková, M., Chambers, J. N., Kumpošt, J., Hubálková, P., Prezeau, L., & Blahos, J. (2016). SGIP1 alters internalization and modulates signaling of activated cannabinoid receptor 1 in a biased manner. *Neuropharmacology*, *107*, 201–214. https://doi.org/10.1016/j.neuropharm.2016.03.008

Halici, Z., Dengiz, G. O., Odabasoglu, F., Suleyman, H., Cadirci, E., & Halici, M. (2007). Amiodarone has anti-inflammatory and anti-oxidative properties: An experimental study in rats with carrageenan-induced paw edema. *European Journal of Pharmacology*, *566*(1–3), 215–221. https://doi.org/10.1016/j.ejphar.2007.03.046

Hanani, M., & Spray, D. C. (2020). Emerging importance of satellite glia in nervous system function and dysfunction. *Nature Reviews. Neuroscience*, *21*(9), 485–498. https://doi.org/10.1038/s41583-020-0333-z

Harris, J., Drew, L. J., & Chapman, V. (2000). Spinal anandamide inhibits nociceptive transmission via cannabinoid receptor activation in vivo. *Neuroreport*, *11*(12), 2817–2819. https://doi.org/10.1097/00001756-200008210-00041

Hohmann, A. G., & Herkenham, M. (1999). Cannabinoid receptors undergo axonal flow in sensory nerves. *Neuroscience*, *92*(4), 1171–1175. https://doi.org/10.1016/s0306-4522(99)00220-1

Hohmann, A. G., Martin, W. J., Tsou, K., & Walker, J. M. (1995). Inhibition of noxious stimulus-evoked activity of spinal cord dorsal horn neurons by the cannabinoid WIN 55,212-2. *Life Sciences*, *56*(23–24), 2111–2118. https://doi.org/10.1016/0024-3205(95)00196-d

Hu, P., Bembrick, A. L., Keay, K. A., & McLachlan, E. M. (2007). Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. *Brain, Behavior, and Immunity*, *21*(5), 599–616. https://doi.org/10.1016/j.bbi.2006.10.013

Hwang, J. H., & Yaksh, T. L. (1997). The effect of spinal GABA receptor agonists on tactile allodynia in a surgically-induced neuropathic pain model in the rat. *Pain*, *70*(1), 15–22. https://doi.org/10.1016/s0304-3959(96)03249-6

Inoue, K., & Tsuda, M. (2009). Microglia and neuropathic pain. *Glia*, *57*(14), 1469–1479. https://doi.org/10.1002/glia.20871

Isik, S., Yeman Kiyak, B., Akbayir, R., Seyhali, R., & Arpaci, T. (2023). Microglia Mediated Neuroinflammation in Parkinson's Disease. *Cells*, *12*(7), 1012. https://doi.org/10.3390/cells12071012

Janjic, J. M., Vasudeva, K., Saleem, M., Stevens, A., Liu, L., Patel, S., & Pollock, J. A. (2018). Low-dose NSAIDs reduce pain via macrophage targeted nanoemulsion delivery to neuroinflammation of the sciatic nerve in rat. *Journal of Neuroimmunology*, *318*, 72–79. https://doi.org/10.1016/j.jneuroim.2018.02.010

Jankauskas, S. S., Wong, D. W. L., Bucala, R., Djudjaj, S., & Boor, P. (2019). Evolving complexity of MIF signaling. *Cellular Signalling*, *57*, 76–88. https://doi.org/10.1016/j.cellsig.2019.01.006

Ji, R.-R., Chamessian, A., & Zhang, Y.-Q. (2016). Pain Regulation by Non-neuronal Cells and Inflammation. *Science (New York, N.Y.)*, *354*(6312), 572–577. https://doi.org/10.1126/science.aaf8924

Jiang, Z., Zhu, H., Wang, P., Que, W., Zhong, L., Li, X., & Du, F. (2022). Different subpopulations of regulatory T cells in human autoimmune disease, transplantation, and tumor immunity. *MedComm*, *3*(2), e137. https://doi.org/10.1002/mco2.137

Jin, S.-X., Zhuang, Z.-Y., Woolf, C. J., & Ji, R.-R. (2003). P38 Mitogen-Activated Protein Kinase Is Activated after a Spinal Nerve Ligation in Spinal Cord Microglia and Dorsal Root Ganglion Neurons and Contributes to the Generation of Neuropathic Pain. *The Journal of Neuroscience*, *23*(10), 4017–4022. https://doi.org/10.1523/JNEUROSCI.23-10-04017.2003

Jin, W., Brown, S., Roche, J. P., Hsieh, C., Celver, J. P., Kovoor, A., Chavkin, C., & Mackie, K. (1999). Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *19*(10), 3773–3780. https://doi.org/10.1523/JNEUROSCI.19-10-03773.1999

Jung, K.-M., Sepers, M., Henstridge, C. M., Lassalle, O., Neuhofer, D., Martin, H., Ginger, M., Frick, A., DiPatrizio, N. V., Mackie, K., Katona, I., Piomelli, D., & Manzoni, O. J. (2012). Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. *Nature Communications*, *3*, 1080. https://doi.org/10.1038/ncomms2045

Kaindl, A., Degos, V., Peineau, S., Gouadon, E., Loron, G., Lombet, A., Verney, C., Mantz, J., & Gressens, P. (2008). Microglia express functional NMDA receptors: A novel finding and a promise for innovative treatment of excitotoxic and inflammatory brain disease. *Neuropediatrics*, *39*. https://doi.org/10.1055/s-0029-1215736

Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S., Egashira, K., & Kasuga, M. (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *The Journal of Clinical Investigation*, *116*(6), 1494–1505. https://doi.org/10.1172/JCI26498

Kandel, E. R. (2013). *Principles of Neural Science, Fifth Edition*. McGraw Hill Professional.

Kano, M., Ohno-Shosaku, T., Hashimotodani, Y., Uchigashima, M., & Watanabe, M. (2009). Endocannabinoid-mediated control of synaptic transmission. *Physiological Reviews*, *89*(1), 309–380. https://doi.org/10.1152/physrev.00019.2008

Kiguchi, N., Maeda, T., Kobayashi, Y., Fukazawa, Y., & Kishioka, S. (2010). Macrophage inflammatory protein-1alpha mediates the development of neuropathic pain following peripheral nerve injury through interleukin-1beta up-regulation. *Pain*, *149*(2), 305–315. https://doi.org/10.1016/j.pain.2010.02.025

Kim, C. F., & Moalem-Taylor, G. (2011). Detailed characterization of neuro-immune responses following neuropathic injury in mice. *Brain Research*, *1405*, 95–108. https://doi.org/10.1016/j.brainres.2011.06.022

Klusáková, I., & Dubový, P. (2009). Experimental models of peripheral neuropathic pain based on traumatic nerve injuries—An anatomical perspective. *Annals of Anatomy = Anatomischer Anzeiger: Official Organ of the Anatomische Gesellschaft*, *191*(3), 248–259. https://doi.org/10.1016/j.aanat.2009.02.007

Kobayashi, Y., Kiguchi, N., Fukazawa, Y., Saika, F., Maeda, T., & Kishioka, S. (2015). Macrophage-T cell interactions mediate neuropathic pain through the glucocorticoid-induced tumor necrosis factor ligand system. *The Journal of Biological Chemistry*, *290*(20), 12603–12613. https://doi.org/10.1074/jbc.M115.636506

Koda, M., Nishio, Y., Hashimoto, M., Kamada, T., Koshizuka, S., Yoshinaga, K., Onodera, S., Nishihira, J., Moriya, H., & Yamazaki, M. (2004). Up-regulation of macrophage migration-inhibitory factor expression after compression-induced spinal cord injury in rats. *Acta Neuropathologica*, *108*(1), 31–36. https://doi.org/10.1007/s00401-004-0853-z

Kohnz, R., & Nomura, D. K. (2014). Chemical Approaches to Therapeutically Target the Metabolism and Signaling of the Endocannabinoid 2-AG and Eicosanoids. *Chemical Society reviews*, *43*(19), 6859–6869. https://doi.org/10.1039/c4cs00047a

Kosersky, D. S., Dewey, W. L., & Harris, L. S. (1973). Antipyretic, analgesic and anti-inflammatory effects of delta 9-tetrahydrocannabinol in the rat. *European Journal of Pharmacology*, *24*(1), 1–7. https://doi.org/10.1016/0014-2999(73)90107-6

Kress, M., & Reeh, P. W. (1996). More sensory competence for nociceptive neurons in culture. *Proceedings of the National Academy of Sciences*, *93*(26), 14995–14997. https://doi.org/10.1073/pnas.93.26.14995

Kuner, R., & Kuner, T. (2021). Cellular Circuits in the Brain and Their Modulation in Acute and Chronic Pain. *Physiological Reviews*, *101*(1), 213–258. https://doi.org/10.1152/physrev.00040.2019

Latremoliere, A., & Woolf, C. J. (2009). Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. *The journal of pain : official journal of the American Pain Society*, *10*(9), 895–926. https://doi.org/10.1016/j.jpain.2009.06.012

Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., Chen, Y., Mitchell, R. A., & Bucala, R. (2003). MIF signal transduction initiated by binding to CD74. *The Journal of Experimental Medicine*, *197*(11), 1467–1476. https://doi.org/10.1084/jem.20030286

Leskovar, A., Turek, J., & Borgens, R. B. (2001). Giant multinucleated macrophages occur in acute spinal cord injury. *Cell and Tissue Research*, *304*(2), 311–315. https://doi.org/10.1007/s004410000325

Liu, Q., Wang, X., & Yi, S. (2018). Pathophysiological Changes of Physical Barriers of Peripheral Nerves After Injury. *Frontiers in Neuroscience*, *12*, 597. https://doi.org/10.3389/fnins.2018.00597

Liu, T., van Rooijen, N., & Tracey, D. J. (2000). Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain*, *86*(1–2), 25–32. https://doi.org/10.1016/s0304-3959(99)00306-1

Loeser, J. D., & Treede, R.-D. (2008). The Kyoto protocol of IASP Basic Pain Terminology. *Pain*, *137*(3), 473–477. https://doi.org/10.1016/j.pain.2008.04.025

Lu, H.-C., & Mackie, K. (2021). Review of the Endocannabinoid System. *Biological Psychiatry. Cognitive Neuroscience and Neuroimaging*, *6*(6), 607–615. https://doi.org/10.1016/j.bpsc.2020.07.016

Lubetsky, J. B., Dios, A., Han, J., Aljabari, B., Ruzsicska, B., Mitchell, R., Lolis, E., & Al-Abed, Y. (2002). The tautomerase active site of macrophage migration inhibitory factor is a potential target for discovery of novel anti-inflammatory agents. *The Journal of Biological Chemistry*, *277*(28), 24976–24982. https://doi.org/10.1074/jbc.M203220200

Luo, C., Kumamoto, E., Furue, H., Chen, J., & Yoshimura, M. (2002). Anandamide inhibits excitatory transmission to rat substantia gelatinosa neurones in a manner different from that of capsaicin. *Neuroscience Letters*, *321*(1), 17–20. https://doi.org/10.1016/S0304-3940(01)02471-5

Mackie, K., Lai, Y., Westenbroek, R., & Mitchell, R. (1995). Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *15*(10), 6552–6561. https://doi.org/10.1523/JNEUROSCI.15-10-06552.1995

Mao, J., Price, D. D., Lu, J., Keniston, L., & Mayer, D. J. (2000). Two distinctive antinociceptive systems in rats with pathological pain. *Neuroscience Letters*, *280*(1), 13–16. https://doi.org/10.1016/s0304-3940(99)00998-2

Mapplebeck, J. C. S., Beggs, S., & Salter, M. W. (2016). Sex differences in pain: A tale of two immune cells. *Pain*, *157 Suppl 1*, S2–S6. https://doi.org/10.1097/j.pain.0000000000000389

Martin, W. J., Hohmann, A. G., & Walker, J. M. (1996). Suppression of Noxious Stimulus-Evoked Activity in the Ventral Posterolateral Nucleus of the Thalamus by a Cannabinoid Agonist: Correlation between Electrophysiological and Antinociceptive Effects. *The Journal of Neuroscience*, *16*(20), 6601–6611. https://doi.org/10.1523/JNEUROSCI.16-20-06601.1996

Matei, D., Trofin, D., Iordan, D. A., Onu, I., Condurache, I., Ionite, C., & Buculei, I. (2023). The Endocannabinoid System and Physical Exercise. *International Journal of Molecular Sciences*, *24*(3), 1989. https://doi.org/10.3390/ijms24031989

Mellor, A. (2012). Fundamentals of Inflammation edited by Charles N. Serhan, Peter A. Ward, and Derek W. Gilroy. *The Quarterly Review of Biology*, *87*(1), 77–77. https://doi.org/10.1086/663914

Melzack, R., & Wall, P. D. (1965). Pain mechanisms: A new theory. *Science (New York, N.Y.)*, *150*(3699), 971–979. https://doi.org/10.1126/science.150.3699.971

Michalski, J.-P., & Kothary, R. (2015). Oligodendrocytes in a Nutshell. *Frontiers in Cellular Neuroscience*, *9*, 340. https://doi.org/10.3389/fncel.2015.00340

Millan, M. J. (1999). The induction of pain: An integrative review. *Progress in Neurobiology*, *57*(1), 1–164. https://doi.org/10.1016/s0301-0082(98)00048-3

Millan, M. J. (2002). Descending control of pain. *Progress in Neurobiology*, *66*(6), 355–474. https://doi.org/10.1016/s0301-0082(02)00009-6

Mitchell, R. A., Liao, H., Chesney, J., Fingerle-Rowson, G., Baugh, J., David, J., & Bucala, R. (2002). Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: Regulatory role in the innate immune response. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(1), 345–350. https://doi.org/10.1073/pnas.012511599

Mitsi, V., & Zachariou, V. (2016). Modulation of pain, nociception, and analgesia by the brain reward center. *Neuroscience*, *338*, 81–92. https://doi.org/10.1016/j.neuroscience.2016.05.017

Moalem, G., Xu, K., & Yu, L. (2004). T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. *Neuroscience*, *129*(3), 767–777. https://doi.org/10.1016/j.neuroscience.2004.08.035

Moore, C. A. C., Milano, S. K., & Benovic, J. L. (2007). Regulation of receptor trafficking by GRKs and arrestins. *Annual Review of Physiology*, *69*, 451–482. https://doi.org/10.1146/annurev.physiol.69.022405.154712

Moore, K. A., Kohno, T., Karchewski, L. A., Scholz, J., Baba, H., & Woolf, C. J. (2002). Partial Peripheral Nerve Injury Promotes a Selective Loss of GABAergic Inhibition in the Superficial Dorsal Horn of the Spinal Cord. *The Journal of Neuroscience*, *22*(15), 6724–6731. https://doi.org/10.1523/JNEUROSCI.22-15-06724.2002

Morisset, V., Ahluwalia, J., Nagy, I., & Urban, L. (2001). Possible mechanisms of cannabinoid-induced antinociception in the spinal cord. *European Journal of Pharmacology*, *429*(1–3), 93–100. https://doi.org/10.1016/s0014-2999(01)01309-7

Moulton, V. R. (2018). Sex Hormones in Acquired Immunity and Autoimmune Disease. *Frontiers in Immunology*, *9*, 2279. https://doi.org/10.3389/fimmu.2018.02279

Moyse, E., Krantic, S., Djellouli, N., Roger, S., Angoulvant, D., Debacq, C., Leroy, V., Fougere, B., & Aidoud, A. (2022). Neuroinflammation: A Possible Link Between Chronic Vascular Disorders and Neurodegenerative Diseases. *Frontiers in Aging Neuroscience*, *14*, 827263. https://doi.org/10.3389/fnagi.2022.827263

Munro, S., Thomas, K. L., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, *365*(6441), 61–65. https://doi.org/10.1038/365061a0

Murata, Y., Onda, A., Rydevik, B., Takahashi, I., Takahashi, K., & Olmarker, K. (2006). Changes in pain behavior and histologic changes caused by application of tumor necrosis factor-alpha to the dorsal root ganglion in rats. *Spine*, *31*(5), 530–535. https://doi.org/10.1097/01.brs.0000201260.10082.23

Muzio, L., Viotti, A., & Martino, G. (2021). Microglia in Neuroinflammation and Neurodegeneration: From Understanding to Therapy. *Frontiers in Neuroscience*, *15*, 742065. https://doi.org/10.3389/fnins.2021.742065

Nathan, C. (2006). Neutrophils and immunity: Challenges and opportunities. *Nature Reviews. Immunology*, *6*(3), 173–182. https://doi.org/10.1038/nri1785

Nathan, C. F. (1987). Secretory products of macrophages. *The Journal of Clinical Investigation*, *79*(2), 319–326. https://doi.org/10.1172/JCI112815

Nerandzic, V., Mrozkova, P., Adamek, P., Spicarova, D., Nagy, I., & Palecek, J. (2018). Peripheral inflammation affects modulation of nociceptive synaptic transmission in the spinal cord induced by N-arachidonoylphosphatidylethanolamine. *British Journal of Pharmacology*, *175*(12), 2322–2336. https://doi.org/10.1111/bph.13849

Nishio, Y., Koda, M., Hashimoto, M., Kamada, T., Koshizuka, S., Yoshinaga, K., Onodera, S., Nishihira, J., Okawa, A., & Yamazaki, M. (2009). Deletion of macrophage migration inhibitory factor attenuates neuronal death and promotes functional recovery after compression-induced spinal cord injury in mice. *Acta Neuropathologica*, *117*(3), 321–328. https://doi.org/10.1007/s00401-008-0476-x

Oprée, A., & Kress, M. (2000). Involvement of the Proinflammatory Cytokines Tumor Necrosis Factor-α, IL-1β, and IL-6 But Not IL-8 in the Development of Heat Hyperalgesia: Effects on Heat-Evoked Calcitonin Gene-Related Peptide Release from Rat Skin. *The Journal of Neuroscience*, *20*(16), 6289–6293. https://doi.org/10.1523/JNEUROSCI.20-16-06289.2000

Ossipov, M. H., Morimura, K., & Porreca, F. (2014). Descending pain modulation and chronification of pain. *Current Opinion in Supportive and Palliative Care*, *8*(2), 143–151. https://doi.org/10.1097/SPC.0000000000000055

Pařízek, A., Suchopár, J., Laštůvka, Z., Alblová, M., Hill, M., & Dušková, M. (2023). The Endocannabinoid System and Its Relationship to Human Reproduction. *Physiological Research*, *72*(S4), S365–S380. https://doi.org/10.33549/physiolres.935229

Perkins, N. M., & Tracey, D. J. (2000). Hyperalgesia due to nerve injury: Role of neutrophils. *Neuroscience*, *101*(3), 745–757. https://doi.org/10.1016/s0306-4522(00)00396-1

Pontearso, M., Slepicka, J., Bhattacharyya, A., Spicarova, D., & Palecek, J. (2024). Dual effect of anandamide on spinal nociceptive transmission in control and inflammatory conditions. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *173*, 116369. https://doi.org/10.1016/j.biopha.2024.116369

Popiolek-Barczyk, K., Kolosowska, N., Piotrowska, A., Makuch, W., Rojewska, E., Jurga, A. M., Pilat, D., & Mika, J. (2015). Parthenolide Relieves Pain and Promotes M2 Microglia/Macrophage Polarization in Rat Model of Neuropathy. *Neural Plasticity*, *2015*, 676473. https://doi.org/10.1155/2015/676473

Pottorf, T., Rotterman, T., McCallum, W., Haley-Johnson, Z., & Alvarez, F. (2022). The Role of Microglia in Neuroinflammation of the Spinal Cord After Peripheral Nerve Injury. *Cells*, *11*(13). https://doi.org/10.3390/cells11132083

Qin, J., Ma, Z., Chen, X., & Shu, S. (2023). Microglia activation in central nervous system disorders: A review of recent mechanistic investigations and development efforts. *Frontiers in Neurology*, *14*, 1103416. https://doi.org/10.3389/fneur.2023.1103416

Raichlen, D. A., Foster, A. D., Gerdeman, G. L., Seillier, A., & Giuffrida, A. (2012). Wired to run: Exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the «runner's high». *The Journal of Experimental Biology*, *215*(Pt 8), 1331–1336. https://doi.org/10.1242/jeb.063677

Raja, S. N., Carr, D. B., Cohen, M., Finnerup, N. B., Flor, H., Gibson, S., Keefe, F. J., Mogil, J. S., Ringkamp, M., Sluka, K. A., Song, X.-J., Stevens, B., Sullivan, M. D., Tutelman, P. R.,

Ushida, T., & Vader, K. (2020). The revised International Association for the Study of Pain definition of pain: Concepts, challenges, and compromises. *Pain*, *161*(9), 1976–1982. https://doi.org/10.1097/j.pain.0000000000001939

Ransohoff, R. M., Schafer, D., Vincent, A., Blachère, N. E., & Bar-Or, A. (2015). Neuroinflammation: Ways in Which the Immune System Affects the Brain. *Neurotherapeutics*, *12*(4), 896–909. https://doi.org/10.1007/s13311-015-0385-3

Ren, K., & Dubner, R. (2010). Interactions between the immune and nervous systems in pain. *Nature Medicine*, *16*(11), 1267–1276. https://doi.org/10.1038/nm.2234

Rexed, B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. *The Journal of Comparative Neurology*, *96*(3), 414–495. https://doi.org/10.1002/cne.900960303

Richardson, J. D., Aanonsen, L., & Hargreaves, K. M. (1998a). Antihyperalgesic effects of spinal cannabinoids. *European Journal of Pharmacology*, *345*(2), 145–153. https://doi.org/10.1016/s0014-2999(97)01621-x

Richardson, J. D., Aanonsen, L., & Hargreaves, K. M. (1998b). Hypoactivity of the spinal cannabinoid system results in NMDA-dependent hyperalgesia. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *18*(1), 451–457. https://doi.org/10.1523/JNEUROSCI.18-01-00451.1998

Ronchetti, D., Borghi, V., Gaitan, G., Herrero, J. F., & Impagnatiello, F. (2009). NCX 2057, a novel NO-releasing derivative of ferulic acid, suppresses inflammatory and nociceptive responses in in vitro and in vivo models. *British Journal of Pharmacology*, *158*(2), 569–579. https://doi.org/10.1111/j.1476-5381.2009.00324.x

Rosen, S., Ham, B., & Mogil, J. S. (2017a). Sex differences in neuroimmunity and pain. *Journal of Neuroscience Research*, *95*(1–2), 500–508. https://doi.org/10.1002/jnr.23831

Rosen, S., Ham, B., & Mogil, J. S. (2017b). Sex differences in neuroimmunity and pain. *Journal of Neuroscience Research*, *95*(1–2), 500–508. https://doi.org/10.1002/jnr.23831

Roved, J., Westerdahl, H., & Hasselquist, D. (2017). Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. *Hormones and Behavior*, *88*, 95–105. https://doi.org/10.1016/j.yhbeh.2016.11.017

Sandkühler, J. (2009). Models and mechanisms of hyperalgesia and allodynia. *Physiological Reviews*, *89*(2), 707–758. https://doi.org/10.1152/physrev.00025.2008

Schweitzer, P. (2000). Cannabinoids decrease the K(+) M-current in hippocampal CA1 neurons. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *20*(1), 51–58. https://doi.org/10.1523/JNEUROSCI.20-01-00051.2000

Shaw, S. K., Owolabi, S. A., Bagley, J., Morin, N., Cheng, E., LeBlanc, B. W., Kim, M., Harty, P., Waxman, S. G., & Saab, C. Y. (2008). Activated polymorphonuclear cells promote injury and excitability of dorsal root ganglia neurons. *Experimental Neurology*, *210*(2), 286–294. https://doi.org/10.1016/j.expneurol.2007.11.024

Shen, M., Piser, T. M., Seybold, V. S., & Thayer, S. A. (1996). Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *The Journal of*

Neuroscience: The Official Journal of the Society for Neuroscience, *16*(14), 4322–4334. https://doi.org/10.1523/JNEUROSCI.16-14-04322.1996

Shepherd, A. J., Mickle, A. D., Golden, J. P., Mack, M. R., Halabi, C. M., Kloet, A. D. de, Samineni, V. K., Kim, B. S., Krause, E. G., Robert W. Gereau, I. V., & Mohapatra, D. P. (2018). Macrophage angiotensin II type 2 receptor triggers neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(34), E8057. https://doi.org/10.1073/pnas.1721815115

Shi, X., Leng, L., Wang, T., Wang, W., Du, X., Li, J., McDonald, C., Chen, Z., Murphy, J. W., Lolis, E., Noble, P., Knudson, W., & Bucala, R. (2006). CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity*, *25*(4), 595–606. https://doi.org/10.1016/j.immuni.2006.08.020

Sommer, C. (2004). Serotonin in pain and analgesia: Actions in the periphery. *Molecular Neurobiology*, *30*(2), 117–125. https://doi.org/10.1385/MN:30:2:117

Sommer, C., & Kress, M. (2004). Recent findings on how proinflammatory cytokines cause pain: Peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neuroscience Letters*, *361*(1–3), 184–187. https://doi.org/10.1016/j.neulet.2003.12.007

Sorge, R. E., Mapplebeck, J. C. S., Rosen, S., Beggs, S., Taves, S., Alexander, J. K., Martin, L. J., Austin, J.-S., Sotocinal, S. G., Chen, D., Yang, M., Shi, X. Q., Huang, H., Pillon, N. J., Bilan, P. J., Tu, Y., Klip, A., Ji, R.-R., Zhang, J., … Mogil, J. S. (2015a). Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature Neuroscience*, *18*(8), 1081–1083. https://doi.org/10.1038/nn.4053

Sorge, R. E., Mapplebeck, J. C. S., Rosen, S., Beggs, S., Taves, S., Alexander, J. K., Martin, L. J., Austin, J.-S., Sotocinal, S. G., Chen, D., Yang, M., Shi, X. Q., Huang, H., Pillon, N. J., Bilan, P. J., Tu, Y., Klip, A., Ji, R.-R., Zhang, J., … Mogil, J. S. (2015b). Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature Neuroscience*, *18*(8), 1081–1083. https://doi.org/10.1038/nn.4053

Sorge, R. E., & Totsch, S. K. (2017). Sex Differences in Pain. *Journal of Neuroscience Research*, *95*(6), 1271–1281. https://doi.org/10.1002/jnr.23841

Spicarova, D., Nerandzic, V., Muzik, D., Pontearso, M., Bhattacharyya, A., Nagy, I., & Palecek, J. (2023). Inhibition of synaptic transmission by anandamide precursor 20:4-NAPE is mediated by TRPV1 receptors under inflammatory conditions. *Frontiers in Molecular Neuroscience*, *16*, 1188503. https://doi.org/10.3389/fnmol.2023.1188503

Spicarova, D., Nerandzic, V., & Palecek, J. (2011). Modulation of spinal cord synaptic activity by tumor necrosis factor α in a model of peripheral neuropathy. *Journal of Neuroinflammation*, *8*, 177. https://doi.org/10.1186/1742-2094-8-177

Spicarova, D., & Palecek, J. (2009). The role of the TRPV1 endogenous agonist N-Oleoyldopamine in modulation of nociceptive signaling at the spinal cord level. *Journal of Neurophysiology*, *102*(1), 234–243. https://doi.org/10.1152/jn.00024.2009

Spiller, K. J., Bi, G., He, Y., Galaj, E., Gardner, E. L., & Xi, Z. (2019). Cannabinoid CB1 and CB2 receptor mechanisms underlie cannabis reward and aversion in rats. *British Journal of Pharmacology*, *176*(9), 1268–1281. https://doi.org/10.1111/bph.14625

Ss, N., Ml, M., Dh, H., & Aj, M. (2013). Fatty Acid modulation of the endocannabinoid system and the effect on food intake and metabolism. *International Journal of Endocrinology*, *2013*. https://doi.org/10.1155/2013/361895

Steeds, C. E. (2016). The anatomy and physiology of pain. *Surgery (Oxford)*, *34*(2), 55–59. https://doi.org/10.1016/j.mpsur.2015.11.005

Stella, N. (2010). Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia*, *58*(9), 1017–1030. https://doi.org/10.1002/glia.20983

Stoll, G., & Müller, H. W. (1999). Nerve injury, axonal degeneration and neural regeneration: Basic insights. *Brain Pathology (Zurich, Switzerland)*, *9*(2), 313–325. https://doi.org/10.1111/j.1750-3639.1999.tb00229.x

Strle, K., Zhou, J. H., Shen, W. H., Broussard, S. R., Johnson, R. W., Freund, G. G., Dantzer, R., & Kelley, K. W. (2001). Interleukin-10 in the brain. *Critical Reviews in Immunology*, *21*(5), 427–449.

Su, Y., Wang, Y., Zhou, Y., Zhu, Z., Zhang, Q., Zhang, X., Wang, W., Gu, X., Guo, A., & Wang, Y. (2016). Macrophage migration inhibitory factor activates inflammatory responses of astrocytes through interaction with CD74 receptor. *Oncotarget*, *8*(2), 2719–2730. https://doi.org/10.18632/oncotarget.13739

Sweitzer, S. M., White, K. A., Dutta, C., & DeLeo, J. A. (2002). The differential role of spinal MHC class II and cellular adhesion molecules in peripheral inflammatory versus neuropathic pain in rodents. *Journal of Neuroimmunology*, *125*(1–2), 82–93. https://doi.org/10.1016/s0165-5728(02)00036-x

Taylor, B. K. (2009). Spinal inhibitory neurotransmission in neuropathic pain. *Current Pain and Headache Reports*, *13*(3), 208–214. https://doi.org/10.1007/s11916-009-0035-8

Tillmann, S., Bernhagen, J., & Noels, H. (2013). Arrest Functions of the MIF Ligand/Receptor Axes in Atherogenesis. *Frontiers in Immunology*, *4*. https://doi.org/10.3389/fimmu.2013.00115

Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nature reviews. Neuroscience*, *11*(12), 823–836. https://doi.org/10.1038/nrn2947

Todd, A. J., & Spike, R. C. (1993). The localization of classical transmitters and neuropeptides within neurons in laminae I–III of the mammalian spinal dorsal horn. *Progress in Neurobiology*, *41*(5), 609–645. https://doi.org/10.1016/0301-0082(93)90045-T

Torres-Méndez, R., Acuña-Prats, R., Salazar-Trujillo, N. V., Cruz, S. L., & Molina-Covarrubias, J. C. (2022). Opioids in Pain. In S. L. Cruz (A c. Di), *Opioids: Pharmacology, Abuse, and Addiction* (pp. 197–223). Springer International Publishing. https://doi.org/10.1007/978-3-031-09936-6_10

Tracey, W. D. (2017). Nociception. *Current Biology: CB*, *27*(4), R129–R133. https://doi.org/10.1016/j.cub.2017.01.037

Treede, R. D., Kenshalo, D. R., Gracely, R. H., & Jones, A. K. (1999). The cortical representation of pain. *Pain*, *79*(2–3), 105–111. https://doi.org/10.1016/s0304-3959(98)00184-5

Treede, R.-D., Rief, W., Barke, A., Aziz, Q., Bennett, M. I., Benoliel, R., Cohen, M., Evers, S., Finnerup, N. B., First, M. B., Giamberardino, M. A., Kaasa, S., Korwisi, B., Kosek, E., Lavand'homme, P., Nicholas, M., Perrot, S., Scholz, J., Schug, S., … Wang, S.-J. (2019). Chronic pain as a symptom or a disease: The IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). *PAIN*, *160*(1), 19. https://doi.org/10.1097/j.pain.0000000000001384

Troiani, F., Nikolic, K., & Constandinou, T. G. (2018). Simulating optical coherence tomography for observing nerve activity: A finite difference time domain bi-dimensional model. *PLOS ONE*, *13*(7), e0200392. https://doi.org/10.1371/journal.pone.0200392

Tsou, K., Lowitz, K. A., Hohmann, A. G., Martin, W. J., Hathaway, C. B., Bereiter, D. A., & Walker, J. M. (1996). Suppression of noxious stimulus-evoked expression of Fos protein-like immunoreactivity in rat spinal cord by a selective cannabinoid agonist. *Neuroscience*, *70*(3), 791–798. https://doi.org/10.1016/s0306-4522(96)83015-6

Twitchell, W., Brown, S., & Mackie, K. (1997). Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *Journal of Neurophysiology*, *78*(1), 43–50. https://doi.org/10.1152/jn.1997.78.1.43

Uçeyler, N., Eberle, T., Rolke, R., Birklein, F., & Sommer, C. (2007). Differential expression patterns of cytokines in complex regional pain syndrome. *Pain*, *132*(1–2), 195–205. https://doi.org/10.1016/j.pain.2007.07.031

Uçeyler, N., Rogausch, J. P., Toyka, K. V., & Sommer, C. (2007). Differential expression of cytokines in painful and painless neuropathies. *Neurology*, *69*(1), 42–49. https://doi.org/10.1212/01.wnl.0000265062.92340.a5

Vacca, V., Marinelli, S., De Angelis, F., Angelini, D. F., Piras, E., Battistini, L., Pavone, F., & Coccurello, R. (2021). Sexually Dimorphic Immune and Neuroimmune Changes Following Peripheral Nerve Injury in Mice: Novel Insights for Gender Medicine. *International Journal of Molecular Sciences*, *22*(9), 4397. https://doi.org/10.3390/ijms22094397

Vacca, V., Marinelli, S., Pieroni, L., Urbani, A., Luvisetto, S., & Pavone, F. (2014). Higher pain perception and lack of recovery from neuropathic pain in females: A behavioural, immunohistochemical, and proteomic investigation on sex-related differences in mice. *Pain*, *155*(2), 388–402. https://doi.org/10.1016/j.pain.2013.10.027

Vacca, V., Marinelli, S., Pieroni, L., Urbani, A., Luvisetto, S., & Pavone, F. (2016). 17beta-estradiol counteracts neuropathic pain: A behavioural, immunohistochemical, and proteomic investigation on sex-related differences in mice. *Scientific Reports*, *6*, 18980. https://doi.org/10.1038/srep18980

Vergne-Salle, P., & Bertin, P. (2021). Chronic pain and neuroinflammation. *Joint Bone Spine*, *88*(6), 105222. https://doi.org/10.1016/j.jbspin.2021.105222

Wall, P. D., McMahon, S. B., & Koltzenburg, M. (2006). *Wall and Melzack's Textbook of Pain*. Elsevier/Churchill Livingstone.

Wang, F., Shen, X., Guo, X., Peng, Y., Liu, Y., Xu, S., & Yang, J. (2010). Spinal macrophage migration inhibitory factor contributes to the pathogenesis of inflammatory hyperalgesia in rats. *Pain*, *148*(2), 275–283. https://doi.org/10.1016/j.pain.2009.11.011

Wang, F., Xu, S., Shen, X., Guo, X., Peng, Y., & Yang, J. (2011). Spinal macrophage migration inhibitory factor is a major contributor to rodent neuropathic pain-like hypersensitivity. *Anesthesiology*, *114*(3), 643–659. https://doi.org/10.1097/ALN.0b013e31820a4bf3

Wang, X., Ma, S., Wu, H., Shen, X., Xu, S., Guo, X., Bolick, M. L., Wu, S., & Wang, F. (2018). Macrophage migration inhibitory factor mediates peripheral nerve injury-induced hypersensitivity by curbing dopaminergic descending inhibition. *Experimental & Molecular Medicine*, *50*(2), e445. https://doi.org/10.1038/emm.2017.271

Watkins, L. R., Nguyen, K. T., Lee, J. E., & Maier, S. F. (1999). Dynamic regulation of proinflammatory cytokines. *Advances in Experimental Medicine and Biology*, *461*, 153–178. https://doi.org/10.1007/978-0-585-37970-8_10

Willis, W. D., & Westlund, K. N. (1997). Neuroanatomy of the pain system and of the pathways that modulate pain. *Journal of Clinical Neurophysiology: Official Publication of the American Electroencephalographic Society*, *14*(1), 2–31. https://doi.org/10.1097/00004691-199701000-00002

Woller, S. A., Eddinger, K. A., Corr, M., & Yaksh, T. L. (2017a). An overview of pathways encoding nociception. *Clinical and Experimental Rheumatology*, *35 Suppl 107*(5), 40–46.

Woller, S. A., Eddinger, K. A., Corr, M., & Yaksh, T. L. (2017b). An Overview of Pathways Encoding Nociception. *Clinical and experimental rheumatology*, *35*(Suppl 107), 40–46.

Wu, X., Yan, Y., & Zhang, Q. (2021). Neuroinflammation and Modulation Role of Natural Products After Spinal Cord Injury. *Journal of Inflammation Research*, *14*, 5713–5737. https://doi.org/10.2147/JIR.S329864

Yu, X., Liu, H., Hamel, K. A., Morvan, M. G., Yu, S., Leff, J., Guan, Z., Braz, J. M., & Basbaum, A. I. (2020). Dorsal root ganglion macrophages contribute to both the initiation and persistence of neuropathic pain. *Nature Communications*, *11*(1), 264. https://doi.org/10.1038/s41467-019-13839-2

Zeilhofer, H. U., Wildner, H., & Yévenes, G. E. (2012). Fast synaptic inhibition in spinal sensory processing and pain control. *Physiological Reviews*, *92*(1), 193–235. https://doi.org/10.1152/physrev.00043.2010

Zhang, W., Xiao, D., Mao, Q., & Xia, H. (2023). Role of neuroinflammation in neurodegeneration development. *Signal Transduction and Targeted Therapy*, *8*(1), 1–32. https://doi.org/10.1038/s41392-023-01486-5

Zhao, Y.-N., Wang, F., Fan, Y.-X., Ping, G.-F., Yang, J.-Y., & Wu, C.-F. (2013). Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure. *Behavioural Brain Research*, *236*(1), 270–282. https://doi.org/10.1016/j.bbr.2012.08.052

Zhou, Y., Guo, W., Zhu, Z., Hu, Y., Wang, Y., Zhang, X., Wang, W., Du, N., Song, T., Yang, K., Guan, Z., Wang, Y., & Guo, A. (2018). Macrophage migration inhibitory factor facilitates production of CCL5 in astrocytes following rat spinal cord injury. *Journal of Neuroinflammation*, *15*(1), 253. https://doi.org/10.1186/s12974-018-1297-z

Zuo, Y., Perkins, N. M., Tracey, D. J., & Geczy, C. L. (2003). Inflammation and hyperalgesia induced by nerve injury in the rat: A key role of mast cells. *Pain*, *105*(3), 467–479. https://doi.org/10.1016/S0304-3959(03)00261-6

List of publications related to this doctoral thesis:

Pontearso Monica, Slepicka Jakub, Bhattacharyya Anirban, Spicarova Diana, Palecek Jiri. **Dual effect of anandamide on spinal nociceptive transmission in control and inflammatory conditions.**

Biomed Pharmacother. 2024 Apr;173:116369. doi: 10.1016/j.biopha.2024.116369. **IF=7.4**

Spicarova Diana, Nerandzic Vladimir, Muzik David, Pontearso Monica, Bhattacharyya Anirban, Nagy Istvan, Palecek Jiri.

Inhibition of synaptic transmission by anandamide precursor 20:4-NAPE is mediated by TRPV1 receptors under inflammatory conditions.

Front Mol Neurosci. 2023 Jun 22;16:1188503. doi: 10.3389/fnmol.2023.1188503. **IF=4.8**

MIF antagonist ISO-1 reduced mechanical allodynia and neuroinflammation in nerve injury neuropathic pain model with differential gender effect.

Monica Pontearso, Anirban Bhattacharyya, Daniel Vasconcelos, Pavel Adamek, Jakub Slepicka, Diana Spicarova and Jiri Palecek

under preparation

Deletion of SGIP-1 protein increases cannabinoid receptor activation-mediated inhibition of spinal nociceptive transmission.

Pontearso Monica, Slepicka Jakub, Denissa Dresslerova, Bhattacharyya Anirban, Palecek Jiri and Spicarova Diana

under preparation