UNIVERSITÀ DEGLI STUDI DELL'AQUILA

DIPARTIMENTO DI MEDICINA CLINICA, SANITÀ PUBBLICA, SCIENZE DELLA VITA E DELL'AMBIENTE

Dottorato di Ricerca in Dottorato di Ricerca in Medicina Clinica e Sanità Pubblica

Curriculum Medicina interna, scienze cardiovascolari e dei sistemi

XXXVII ciclo

Titolo della tesi

Evaluation of signaling pathways induced by complement system in the establishment of neuropathic pain: effects of C5aR1 and C5aR2 antagonists

SSD BIO/06

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a.a. 2023/2024

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CHAPTER I

1.1 Abstract

Peripheral neuropathy and hypersensitivity reactions (HSRs), frequently associated with chemotherapy, represent common and debilitating adverse effects linked to the use of paclitaxel as an antineoplastic agent. Evidence indicates that paclitaxel binds to and activates complement component 5a receptors 1 and 2 (C5aR1, C5aR2), a mechanism identified as pivotal in the onset of paclitaxel-induced neuropathy and anaphylaxis. Prior research connecting interleukin-8 (IL-8) to paclitaxel-driven neurotoxicity prompted an investigation into the proteins responsible for IL-8 induction. Molecular docking simulations conducted via the Exscalate platform demonstrated a high binding affinity between paclitaxel and C5aR1 and 2. *In vitro* studies confirmed that the interaction between paclitaxel and C5aR1 is specific and competitive, leading, in particular for C5aR1, to the activation of intracellular NFκB/P38 signaling and c-Fos pathways. In neuronal F11 cells and rat dorsal root ganglia, C5aR1 blockade reduced neuropathological changes caused by paclitaxel. Similarly, in mice treated with paclitaxel, either genetic deletion of C5aR1 or its pharmacological inhibition significantly ameliorated symptoms of chemotherapy-induced peripheral neuropathy (CIPN), including cold and mechanical allodynia, and minimized chronic pathological alterations in the paw. Furthermore, inhibition of C5aR1 suppressed anaphylactic cytokine release triggered by paclitaxel in macrophages during *in vitro* experiments and reduced the incidence of HSRs in mouse models. These findings collectively identify C5aR1 as a key mediator in the pathogenesis of paclitaxel-induced CIPN and HSRs and highlight its potential as a therapeutic target for mitigating these severe adverse effects.

CHAPTER II

2.1 Pain

Defined as uncomfortable sensory event, pain is considered a defense mechanism which take place as acute pain after a noxious stimulus, secondary insults, homeostasis impairment, and infections [1]. Such subjective experience is the most ancient common human event often associated with potential or real injury, with different elements, such as nociception and cognition, but also social and emotional components collaborating to define it [2,3]. On the other hand, inherited genetic alterations can be responsible for insensitivity, making affected subjects unable to avoid damage and thereby feeling pain [4]. While acute pain cause distress for a defined window of time and insensitivity limit pain feeling, many patients experience pain constantly in time. Indeed, chronic pain is intrinsically tormenting. It is the first reason that brings people receive medical attention and represents a global-scale economic burden [5,6]. From an epidemiological point of view, chronic pain shows a prevalence comprised between 10% and 40% with a really low recovery rate of around 5% [7,8]. Even though pain feeling can be deleterious, especially prolonged over time, it is important for monitoring the clinical profile of a patient. In fact, pain is relevant for linking a primary condition to its secondary outcomes, as pain represent the nexus among social, biological, and psychological elements on which a good diagnosis can be based. However, due to the complexity of such dynamic and troublesome mechanisms patients experience low quality of prognosis and even disability [1]. Moreover, the subjective nature of pain causes different levels sensorial stimulation due to different tolerance among people, underscoring the complex processing which take place in the brain and the involvement of more than biology alone. Indeed, pain can be the source of anxiety, depression, insomnia, and social and psychiatric issues leading to deterioration of the clinical condition of patients [9,10]. The impact of pain over time on affected people can be so great to induce drug abuse and raise the number of suicide [11]. Similarly, patients which undergo cancer treatment often encounter pain as a side effect. Such pain is often impactful, weakening the patients and hindering their survival [12]. Thus, pain relief is an indispensable index for clinical treatment. Even though their effects are limited in time, analgesic drugs represent the base for acute and chronic pain management. However, their wrong usage is a concern worldwide. For instance, evidence of increased analgesic addiction and dependence have been found, causing international concern [13,14].

To date, researchers across the world have garnered information via preclinical and clinical studies dissecting many of the mechanisms involved in pain manifestation, paving the way for the development of new therapeutic strategies. In the following chapters pain pathological characteristics and typologies will be discussed.

2.2 Circuits of pain

As previously described, pain shows a complex nature which reflects a more complex interplay of physiological processes which occurs between central and peripheral nervous systems (CNS; PNS). Many actors are involved in such interplay like molecules, cells, and other structures that collaborate to perform all the tasks necessary to produce and modulate a pain signal. Starting from the peripheral event, the following paragraphs will describe how pain establishes after a pain-inducing event, the ascending transmission, and how the brain senses and modulate it (**Figure 1**).

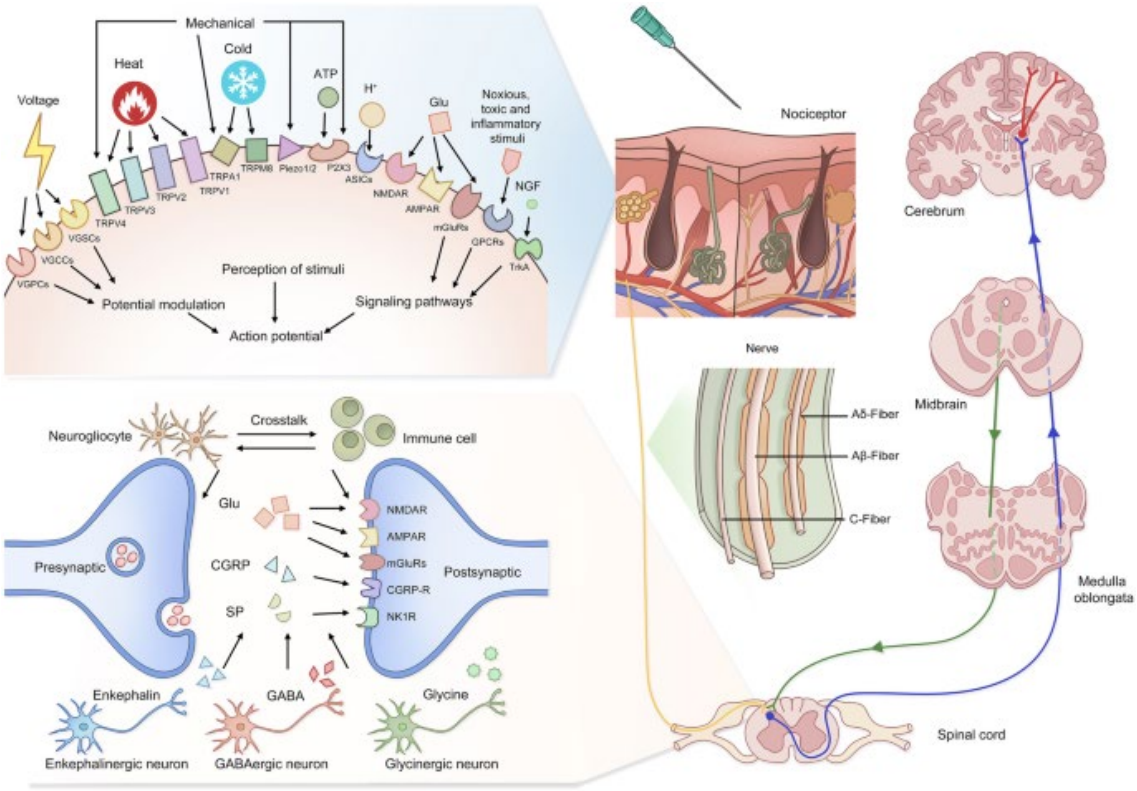


Figure 1: Overview on the basic circuit on pain signals [15].

2.2.1 Peripheral transmission of pain signals

Once a painful event take place, the change in the activity of specific receptors leads to activation of action potential of peripheral nociceptors. Such nociceptors are peripheral pain transducers considered as the initiators of pain sensation and can be classified into the following types according to the nature of the noxious stimulus: mechanical-sensitive, thermo-sensitive, and injury signal-sensitive. In particular, nociceptors are undifferentiated nerve endings located in internal organs as well as muscles, periosteum, mucosa, blood vessels, tendons, and skin. Their soma is located in the trigeminal ganglion and the dorsal root ganglion (DRG). Furthermore, nociceptors show different ion channels on their peripheral end, by which they can sense external stimuli thereby producing membrane excitability. Interestingly, nociceptors are characterized by high thresholds of pain sensation, making it possible for people to perceive tactile stimulation with no pain. Once electric signals are produced by these channels, a chain of events take place. In fact, ion channels across membranes can modulate ion currents, ending in the forced activation of other neighboring ion channels sensitive to changes of voltage. Such channels can be divided into ligand-gated (LG) and voltage-gated (VG) ion channels, depending on the reason behind their activation. The latter type represents a category of transmembrane proteins essential in the electrical activity of these cells. They are characterized by a membrane potential-dependent conformation and are essential in the serial generation of action potentials from the accumulated receptor potential.

Many VG types exists. For example, VG calcium channels (VGCCs) are transmembrane protein present in all those cells with electrical activity. Many factors can modulate VGCCs' activity such as micro RNAs (miRNAs), neuromedin B, and adiponectin [16–18]. Their structure is composed of α1, β1-4, α2δ1-4, and γ1-8 subunits which can change for each VGCC type. In particular, the third subunit plays a relevant role in each VGCC type by strengthening the electrical activity in terms of peak potentials and activation/inactivation rates of the channels. An example of this effect can be found in the establishment of a noxious stimulus which cause increased expression of α2δ in PNS and CNS. The raising levels of α2δ are responsible for enhanced pain sensing [19,20]. Similarly, other players of the pain sensation belong to the family of VG sodium channels (VGSCs): from Nav1.1 to Nav1.9 (*SCN1A* through *SCN5A*, then *SCN8A* through *SCN11A*), these are the nine members of the family. Interestingly, these channels shows a different temporal and spatial distribution, also differing in their biological activity

[21,22]. After cell depolarization, a conformational change for VGSCs cause their opening. Open VGSCs permit sodium exchange in a gradient-dependent matter between extracellular environment and intracellular environment. In particular, sodium influx toward cells is the starting point of spikes formation and pain sensing at the nerve terminals [23]. VGSCs such as Nav1.1, Nav1.6, and Nav1.8 are involved in pain modulation [22,24,25]. However, researchers highlighted the relevance of Nav1.7 in various pain disorders. Indeed, conditions such as small-fiber neuropathy, paroxysmal extreme pain disorder, and erythromelalgia are characterized by mutations in SCN9A, which encodes for Nav1.7 whose strategic inhibition can effectively reduce pain and increase opioids production [26,27]. Nonetheless, Nav1.7's role in pain modulation can vary in each condition: for example, while Nav1.7 is relevant for neuropathic pain establishment, it is less involved in oxaliplatin-induced pain and bone cancer pain [28]. If VGCCs and VGSCs are responsible for raising neuronal excitation, on the other hand VG potassium channels (VGPCs) are involved in its attenuation by inducing hyperpolarization of membranes via potassium efflux from neurons counteracting the ion inward conductance. VGPCs represent the most abundant group of VGs with around 40 related genes and 12 families of hetero- or homo-tetrameric channels. In particular, some subunits (Kv1-Kv4, Kv7, and Kv10-Kv12) are able to form pore structures while other members (Kv5, Kv6, Kv8, and Kv9) can form conductive channels once associated with the pore-forming members. The generation of quaternary structures leads to a great functional diversity, which is increased once other structures are taken into account: splice variants, auxiliary β subunits, and post-translational modifications (PTMs) [29]. Once a noxious stimulus takes place, VGPCs can be downregulated and even inhibited, causing spontaneous firing in nociceptors [30,31].

Regarding the LG ion channels, the transient receptor potential (TRP) channels are the most relevant in this category. TRP channels are widely distributed in CNS and PNS, where they react to chemical and physical stimuli by playing as sensors for itch and pain. Their involvement in pain modulation has been documented for a few of the 28 TRP channels. Indeed, evidence for the modulation of TPV1, TRPV2, TRPV3, TRPV4, TRPA1, and TRPM8 during pain sensation have been found [32]. In particular, TRPV1 and TRPA1 are activated during pain sensation and through them many compounds are able to directly induce pain, suggesting their therapeutic value as key player in pain sensation [33–35]. Oter LG ion channels are the N-methyl-D-aspartate receptors (NMDARs), a family of ionotropic glutamate receptors involved in the excitatory

neurotransmission that are sensitive to mechanical stimulation. NMDARs are characterized by quaternary associations of subunits such as GluN1, GluN2A-D, and GluN3A-B. Each subunit comprise different membrane domains (M1-M4), a large extracellular N-terminal domain composed of a portion of the ligand-binding domain (LBD) and the amino-terminal domain, an intracellular C-terminal domain, and a loop between M3 and M4 placed extracellularly which allocate the second portion of the LBD [36]. Thus, NMDARs are composed of different combinations of GluN subunits, which define their functional characteristics. NMDARs are pivotal in the induction of an electrical signal. They can tonically activate primary afferent neurons by interacting with VGCCs subunit α2δ. Moreover, NMDARs ca be activated neuronal depolarization [37]. Their relevance in pain establishment and maintenance has been described. Indeed, both presynaptic and postsynaptic NMDARs can modulate synaptic plasticity and excitatory synaptic transmission, facilitating hyperalgesia [38,39]. Also, NMDARs are activated by neuropathic pain under continuous endogenous glutamate stimulation, in a vicious cycle which cause and exacerbates chronic pain [40,41].

Nociceptors display a plethora of different channels, different from the ones described so far, which are involved in the conversion of the noxious stimulus into the electrical signal. Among them, it is possible to find bradykinin and nicotinic acetylcholine receptors, Piezo 1/2, calcium-permeable purinergic receptor (P2X) channels, and acid-sensing ion channels (ASICs) [42]. Particularly, the latter are part of the epithelial sodium channel/degenerin family which play a role in hyperalgesia modulation by sensing pH alterations in the extracellular domain during inflammatory events related to cancer or ischemia [43]. Regarding P2X, they are hyperactive receptors for pain sensitization involved in sensing extracellular ATP level variations [44]. Interestingly, P2X3 receptor is suppressed by ASIC3 by forming a complex thanks to cellular co-localization and similarity in molecular structures, suggesting how specific molecular processes work together to regulate or balance pain perception in the body [45]. Other receptors involved in the transduction of the electric signals are the tropomyosin receptor kinases, which interact with neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) to modulate neuronal survival and growth, and G protein-coupled receptors (GPCRs) [46]. Regarding GPCRs, as the largest group of transmembrane proteins they are widely distributed in neurons but also glial and immune cells. GPCRs are involved in the physiological modulation of nociceptive transmission directly regulating ion channels and activating pain-related pathways when activated by

pain-related ligands: for instance, glutamate, cannabinoids, opioids, γ-aminobutyric acid (GABA), histamine, bradykinin, 5-hydroxytryptamine (5-HT) [47,48]. After the ligand-receptor interaction, a conformation change of GPCR's α subunit determines its activation causing modulation of the production of secondary messengers with different effects on pain manifestations, involving intercellular communication, neuronal excitability, neurotransmitter release, and inflammation [49].

After the noxious stimuli, nociceptors produce electrical signals that are directly sent, via nerve afferent fibers, to the spinal cord. Nerve afferent fibers are basically axons of sensory neurons and can be divided, depending on functional and anatomical properties, into different categories: Aα, Aβ, Aδ and C fibers. Regarding A fibers, they are characterized by myelin sheaths to perform a faster transmission of action potentials. In particular, Schwann cells produce myelin sheaths of different diameters (thick, medium, and thin for Aα, Aβ, Aδ fibers respectively) which differently affect the signal speed across the axons [50]. Moreover, Schwann cells are sensitive to noxious stimuli and by exploiting intracellular oxidative stress they can release reactive oxygen species (ROS) to influence the pain events stimulating, for instance, TRPA1 [51,52]. Concerning C fibers, the involvement of Nav1.7 as prerequisite for action potential initiation has been described, highlighting once more the relevance of these channels in pain sensation [27]. Nociceptors belong to Aδ and C fibers. Their stimulation can bring to piercing, fast pain for A fibers whereas the stimulation of C fibers causes burning and poorly-localized pain [53,54]. Overall, each fiber type can be subjected to injury and even inflammatory events with fiber sensitization, ending in decreased pain thresholds and persistent pain manifestation [53].

2.2.2 Ascending pathway for pain transduction

Once a noxious stimulus take place in the periphery, the signals reach and activate multiple neurons in the DRG, the first-order neuron. The bodies of the first-order neurons, within the DRGs, project their axons to the posterior gray horn of the spinal cord where they synapse with second-order neurons that ascend along the spinal cord and project toward third-order neurons found in the subcortical structures of the brain, such as the thalamus. Thus, the signals are received by spinal projection neurons, as second-order neurons, which further process and send them into ascending pathways,

ultimately ending the passage from PNS to CNS [55]. For instance, the trigeminothalamic tract and the spinothalamic tract are pivotal in pain transduction. To transport the pain sensation from face and head, the trigeminothalamic tract exploit pontine trigeminal nuclei and fibers derived from the spinal trigeminal nucleus, both of which are its constituents. On the other hand, the spinothalamic tract, which is responsible for pain transduction from limbs and torso, is diverted in anterior and lateral tracts at the level of the anterior white commissure. In particular, while the anterior spinothalamic tract transports sensory information such as crude touch or pressure to the ventral posterolateral nucleus of the thalamus, the later spinothalamic tract is a bundle of afferent nerve fibers which carries information related to pain and temperature up to the thalamus [56]. Running parallel to the spinothalamic tract, the spinoreticular tract is composed of ascending fibers placed in the anterolateral part of the spinal cord that reach the brainstem reticular formation conveying information the control of descending modulation, motivational-affective aspects of pain, as well as motor and neurovegetative responses to pain [57]. Regarding the spinohypothalamic tract, it projects from the spinal cord directly to the hypothalamus, where, by synapsing with the hypothalamic neurons, the fibers mediate the response of the autonomic nervous system to pain [58]. Other ascending pathways relevant in pain transduction are the spinomesencephalic tract and postsynaptic dorsal column fiber bundles. The former is part of the spinal cord's anterolateral system and comprises a number of ascending tracts in the spinal cord, including the spinotectal tract, projecting to different areas of the midbrain in order to process visceral sensations and pain [59]. Regarding the latter, postsynaptic dorsal column cells are second-order sensory neurons in the spinal cord which receive cutaneous mechanical and/or noxious visceral information from the periphery by primary afferents. Postsynaptic dorsal column fiber bundles transfer the information ascending through the dorsal column to innervate the dorsal column nuclei in the dorsal midline of the caudal medulla. Here, cells located in laminae III and IV primarily propagate information about innocuous cutaneous mechanical stimuli while those located in lamina X transmit information about noxious stimuli received from afferent fibers innervating inflamed visceral organs [60]. Visceral pain pathways of transduction show different characteristics compared to classical circuits. Indeed, these pathways are decentralized, with axons of visceral sensory neurons spreading throughout internal organs and their blood vessels. In particular, the cell bodies of afferent visceral sensory neurons through parasympathetic nerves are located in the sensory ganglia of the

glossopharyngeal nerve and vagus, as well as in the S2-4 spinal ganglion. Those via sympathetic nerves can be found at the T1-L3 spinal ganglion [61].

2.2.3 Pain perception and modulation in the brain

All the elements that actively collaborate to modulate pain signals are coordinated by the CNS, including, interneurons, afferent fibers, and projection neurons, shaping a sort of matrix which is at the base of pain modulation. Such pain matrix comprise CNS' subcortical and cortical regions like hypothalamus, thalamus, periaqueductal gray, parabrachial nuclei, amygdala, nucleus tractus solitarius, somatosensory cortex, prefrontal cortex, insular cortex, and anterior cingulate cortex [62]. At the center of this matrix, the primary somatosensory cortex (S1) receives and organizes noxious stimuli. In particular, S1 receives information from the ventral posterolateral thalamus and transmits the processed information to different subcortical regions [63]. Once a pain stimulus is sent up to the CNS from the periphery, a dramatic increase in S1 activity can be observed, supported by blockage of innate antinociceptive processes and enhancement of thalamocortical signaling by layer 6 neurons [64]. Moreover, the thalamus and the lateral parabrachial nuclei are pivotal in the integration and modulation of nociceptive stimuli transmitted across the spinal cord. Similarly, the central amygdala play an important role in modulating the pathological effects and the behavioral outcomes of noxious stimuli interacting with hypothalamus and brainstem [65]. It is important to state that chronic pain is responsible for central sensitization by altering the metabolic patterns in the involved brain regions and supporting alterations in the sensory circuitry [66,67]. Moreover, given the subjectivity of pain perception many factors can be considered as modifiers of the tuning of this pain matrix: damage degree and other factors such as social, emotional, and environmental elements concur in shaping pain perception. Indeed, cognitive evaluation of threats, degree of focus on pain, the individual character, eventual depressive states, all of them can influence the intensity of pain or even starting its manifestation. Furthermore, pain memory can induce hyperalgesia starting from past painful experiences. Behind these manifestations, pain stimuli received by afferent signals are finely processed by many CNS regions such as S1 but also prefrontal cortex, anterior cingulate cortex, and insular cortex, to give rise to pain perception and affection [15,68]. Among the cited regions, group of neurons in the dorsomedial prefrontal cortex are involved in the regulation of pain chronicity [69]. Their

activity is crucial in the development of pain chronicity and their projections reach amygdala, nucleus accumbens, hypothalamus, and other limbic structures building the connection between chronic pain and emotions [69,70]. Nonetheless, many other circuits been found to contribute directly or indirectly to pain establishment and maintenance, few examples of which are: entorhinal cortex, dopaminergic projections from ventral tegmental area and substantia nigra pars compacta to the nucleus accumbens, and the septo-hippocampal circuit [71–74]. As per the areas described before which are involved in ascending pathways, many areas involved in descending pathways can be found as well, including locus coeruleus, nucleus raphes magnus, periaqueductal gray, rostral ventromedial medulla, lateral reticular nucleus, and nucleus paragigantocellularis. Acting as facilitatory and inhibitory systems, these pathways are balanced in physiology. However, pain establishment creates unbalanced activity in favor of the facilitatory systems which react to the noxious stimuli by lowering the pain threshold [75]. It is important to state that this effect is related to hypersensitivity events and not to standard pain perception [76,77]. The major brain components of the facilitatory system are the rostral ventromedial medulla, the periaqueductal gray, and the anterior cingulate cortex, which are characterized by defined regulatory activities, even though a functional overlap can be observed among them [78]. In particular, these components are modulated by the limbic system, which is in part responsible for the regulation of normal behavior, underscoring once again the relevant role of experience and emotions in pain sensation [79]. Nonetheless, the rostral ventromedial medulla and the periaqueductal gray, as well as part of the pontine dorsolateral reticular formation, are also involved in inhibitory systems regulating the pain sensation through the descending spinal dorsolateral tract. Indeed, different neuron subpopulation can shape the multimodal activities of these nuclei [80,81]. An example of this can be found in the rostral ventral medulla in which coexisting ON- and OFF-cells have been found performing inhibitory and facilitatory descending pathways [82]. Other brain regions involved in pain modulation are, for instance, collected in the Default Mode Network (DMN), an ensemble of functional brain areas active when an individual is at rest which comprises angular gyrus, posterior cingulate cortex, medial temporal lobe, precuneus, and medial prefrontal cortex. DMN supports internally focused cognition with mind-wandering, episodic memory retrieval, and self-reflection. Pain establishment requires cognitive resources causing it to compete with attention-demanding stimuli. Such events are related to DMN dysfunction: impaired connectivity between DMN areas and its enhancement are closely associated

with pain severity [83]. For example, the medial prefrontal cortex shows impaired connectivity with the posterior elements of DMN when there is an increment of high frequency oscillations [84]. Interestingly, evidence of the involvement of DMN in mechanisms related to emotional changes and pain sensation have been found [85]. Indeed, while mind-wandering helps in redirecting the spontaneous attention away from pain by restoring the connectivity between the periaqueductal gray and the DNM, the decoupling between DMN and thalamus is an important process of min mindfulness meditation-induced pain relief [86]. Conversely, negative emotions enhance pain hypersensitivity via DMN modulation during chronic pain [87]. Similarly, acute pain can alter the functional connectivity and oscillatory activity of DMN, suggesting the deep involvement of DNM during pain [88].

2.3 Molecular regulation of pain perception

The molecular regulation of pain perception is a finely modulated interplay of molecular actors (**Figure 2**). Among all, genetic mutations and epigenetic alterations, as well as PTMs, and microbiota operate under precise and dynamic regulation ensuring that the sensation of pain is modulated appropriately in response to various stimuli and conditions and maintaining a balance between protective responses and pathological states [15].

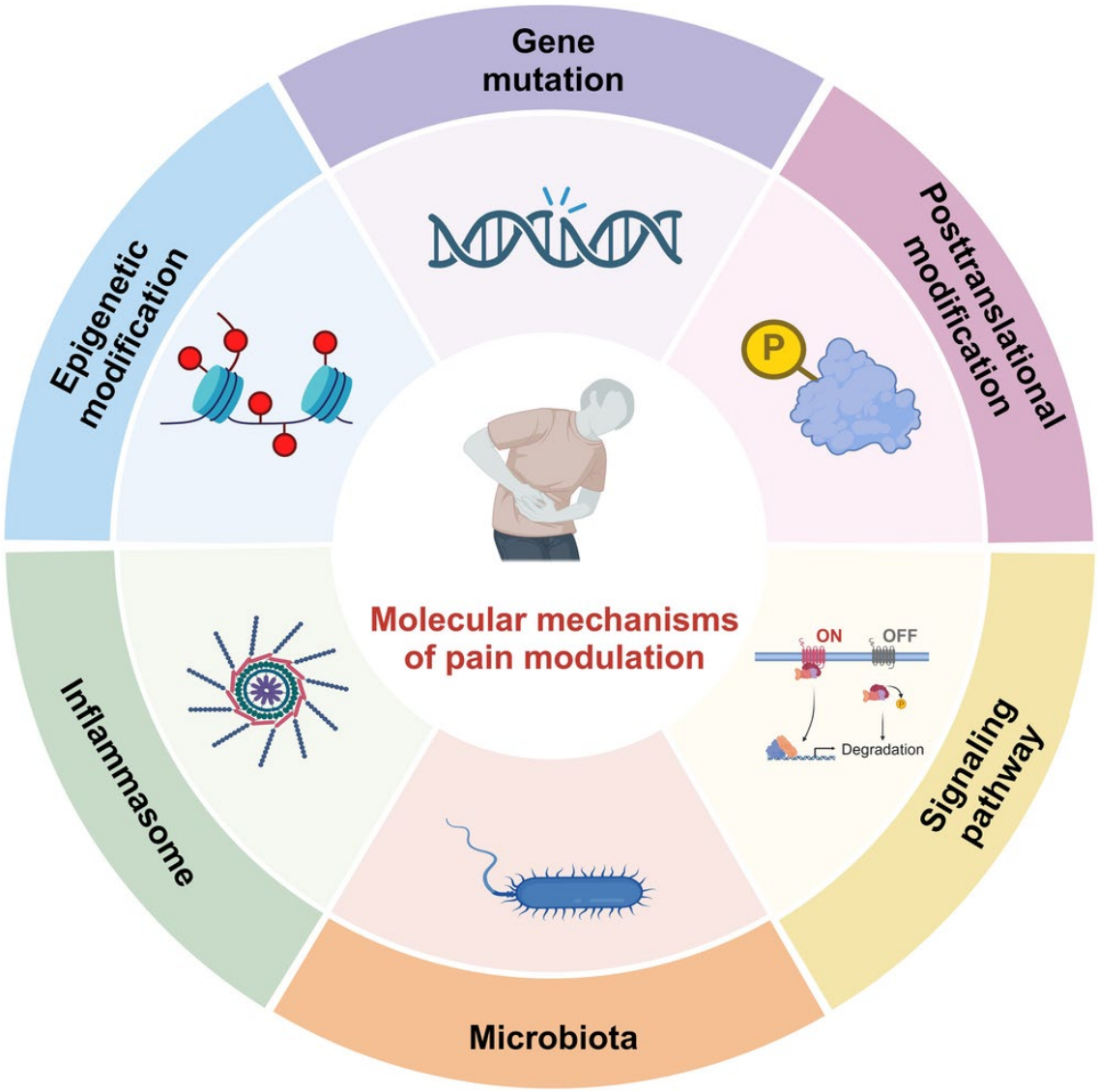


Figure 2: Schematic representation of the major molecular mechanisms involved in pain perception [15]. *While most of them will be dissected in the following sections, neuroinflammation and inflammasome roles will be discussed in the context of CIPN.*

2.3.1 Gene mutations

In pain context, various conditions are defined by identified gene mutations including congenital insensitivity, erythromelalgia, anhidrosis, familial episodic pain syndrome, and Fabry disease. Thus, different mutations can bring totally different clinical outcomes, spanning from pain insensitivity to extreme pain perception, with differences between individuals and mutation patterns [89]. VG ion channels are major actors in this play as well as the most investigated. For instance, missense mutation (L263V) in Nav1.1 gene promote the 5-HT3- and P2X3-dependent spike activity, supporting migraine pain via

enhanced excitability of peripheral trigeminal neurons [90]. Mutations in Nav1.7 gene can lead to function loss or truncation, ending up to congenital pain insensitivity [91]. Interestingly, genetic insights on Nav1.7 demonstrated its involvement in olfaction modulation other than pain, as individuals with mutations in its gene also show anosmia [92,93]. Intronic mutations in *SCN9A*, like the homozygous substitution in intron 3, have been found modulating pain sensitivity by Nav1.7 inactivation due to impaired mRNA splicing [94]. As per *SCN9A*, *SCN11A* can display genetic alterations. Indeed, two missense mutations (c.673 C > T and c.2423 C > G) able to enhance Nav1.9 excitability in DRG sensory neurons have been found related to familial episodic pain syndrome [95]. Again, *SCN11A* shows a mutation at R222S site in patients with cold-sensitive mechanical hyperalgesia [96]. On the other hand, Nav1.9 gene can be subjected in exon 15 to a gain-of-function mutation which brings to loss of pain perception in patients with congenital pain insensitivity due to hyperactivation at resting potential and sustained depolarization of nociceptors [97]. Regarding VGCCs, Cav3.1 can show a missense mutation in position 706 (R706Q) of the *CACNA1G* gene involved in the production of the α1 subunit of the channel, causing increased neuron excitability in the trigeminal neuralgia [98]. Interestingly, a single mutation in α2δ1 is responsible for halting the analgesic action of pregabalin without affecting pain perception, suggesting the role of this subunit in the pregabalin mechanism of action [99]. Similarly, VGPCs can undergo genetic mutations affecting pain perception. For instance, the rare allele SNP rs140124801 in the *KCNG4* gene is responsible for pain attenuation during childbirth due to enhanced potassium outflux and neuron hyperpolarization in uterus [100]. Again, loss of function for the two-pore VGPCs TRESK caused by a frameshift mutation in the gene KCNK8 is responsible for stressed overall hypersensitivity, especially the thermal and mechanical ones, with enhanced neuronal excitability [101]. Lastly, TRP channels have been found to be mutated too. Indeed, TRPV1 can display loss of function when the mutation N331K is present whereas it display a gain of function with G564S variant [102,103]. Moreover, TRPV1 can be alkylated to chemically induce loss of function [104]. Regarding TRPA1, familial episodic pain syndrome is deeply related to the N855S mutation in *TRPA1* gene which cause in activated nociceptors a strong increment in inward current [105,106]. Interestingly, in the TRPA1 gene a non-sense mutation can cause truncation of the TRPV1 channel, conferring hyperactivity when co-expressed with wild-type TRPA1. Indeed, the mutant co-assembles with wild-type TRPA1 subunits

into heteromeric channels in cells that are functional at the plasma membrane reducing the energetic barriers and causing neuronal hyperactivation [107].

Many other genes are targeted by mutations influencing neuronal development and axon growth, and are associated with pain, lastly regulating pain perception with hypo- or hypersensitivity [108–111].

2.3.2 Epigenetic modification

Epigenetics refers to modifications in gene expression which are not embedded in the DNA sequence. Age, environment, and behavior deeply influence via epigenetics the human beings. Epigenetic modification include DNA methylation, histone acetylation and methylation, and non-coding RNAs (**Figure 3**), and are all involved in pain regulation [112]. Regarding DNA methylation, enzymes called DNA methyltransferases (DNMTs) chemically modify the DNA by catalyzing the transfer of a methyl group from S-adenosylmethionine to specific bases in the sequence. DNA methylation mostly occur in distinct sites called CpG islands which are aggregated distributions of bases. Once the methyl groups are transferred, changes in DNA stability and conformation as well as in protein interaction and chromatin structure can be observed. In the pain context, DNA methylation changes in promoter regions have been observed in patients with chronic pain by analyzing global methylation data on immunomodulation, GPCRs signaling, and neurodevelopment [113–115]. During pain, DNA methylation is altered and such alterations can be found early in neuropathic pain but can persist over time [116]. Moreover, differences in DNA methylation can be observed regionally depending on the type of pain, including nerve injury, diabetes neuropathy, and chemotherapy-induced peripheral neuropathy (CIPN). Thus, all these type of neuropathic pain display similar characteristics but differ in epigenetics [117]. In patients with pain disorders, different levels of methylated DNA negatively modulate expression of gene relevant to nociplastic and neuropathic pain perception, among which the calcitonin gene-related peptide, BDNF, and TRPA1 [118–120]. DNMT1, DNMT3A, and DNMT3B are the three DNMTs involved in both CNS and PNS in DNA methylation and neuropathic pain modulation [121]. In particular, methylation of 5'-untranslated region and promoter of *KCNA2A* gene via DNMT1 and DNMT3A can cause poor abundance of VGPCs and decreased current, with central sensitization and neuropathic pain [121,122]. Moreover, the exert regulatory activity by methylating genes related to non-coding RNAs, with detrimental

effects over pain modulation [123,124]. DNMTs involvement in pain perception is deep. Interestingly, their systemic inhibition can ameliorate pain, suggesting hypersensitivity as their overall effect [114]. Nonetheless, DNA methylation can be counteracted by ten-eleven-translocation proteins (TETs), which mediate demethylation of DNA instead. Main members of the TET family are TET1, TET2, and TET3: they are responsible for keeping DNA methylation stable to finely tune the epigenome [125]. In particular, TET1 modulates both positively and negatively pain. Indeed, TET1 is able to exert analgesic activity by promoting VGPCs functioning by changing methylation levels over the promoter of KCNA2 and K2P1.1, as described in models of neuropathic pain [126,127]. Conversely, TET1 can promote by demethylation the expression of gene related to neuropathic pain and nociception progression [128–130]. Regarding histone acetylation, it mainly occurs in H3 and H4, lysine sites, via histone acetyltransferases (HATs) and it is pivotal for regulating histone activity. Histones comprise five families, including H1 and H5 as linker histones, and H2A, H2B, H3, and H4 as core histones. In particular, acetylation of H3 and H4 are dysregulated in CNS and PNS [131,132]. Their acetylation state is upregulated during neuropathic and nociceptive pain establishment in DRG and spinal dorsal horn [133]. Neuroinflammation appeared to be involved in acetylation upregulation as inflammatory mediators such as interleukin (IL) 6 (IL-6), IL-8, and tumor necrosis factor α (TNF-α) enhance the process and promote neuron excitability in neuropathic pain models [134,135]. To counterbalance the effects of histone acetylation the dynamic activity of histone deacetylases (HDACs) is essential. HDACs are divided into four groups and evidence for the association of HDAC1 and HDAC2 with pain sensation have been found. Indeed, downregulated expression of these HDACs is responsible for abnormal synaptic transmission, with visceral and somatic hypersensitivity [19,136,137]. However, the cellular location of such epigenetic modulators is a relevant aspect during pain. For instance, HDAC2 can move to the nucleus under the regulation of the transcription factor Sp1 exasperating microglia-dependent inflammation and neuronal dysregulation [138]. Regarding HDAC3 and HDAC4, while the former is poorly related with pain regulation the latter can exert analgesic activities when its cytoplasmic translocation is inhibited, ending in epigenetic downregulation of the *HMGB1* gene involved in neuropathic pain [137,139]. On the other hand, HDAC5 can accumulate in the nucleus blocking H3 acetylation of two promoters, Gad1 and Gad2, causing aberrant astrogliosis directly via signal transducer and activator of transcription 3 (STAT3) pathway and impaired activity of GABAergic

neurons [140,141]. Other HDACs involved in pain perception are sirtuins 1 and 3 (SIRT1 and SIRT3) which display analgesic effects when downregulated in nervous lesions by alleviating emotional vulnerability of neuropathic pain via reduction of ectopic discharge of sensory neurons [142–144]. Histones can be also affected by methylation. For instance, the methyltransferase EZH2 exploit the site K27 to add a methyl group on H3 [145]. EZH2 is involved in neuropathic pain and cancer pain, as described in rodent models [146,147]. While its upregulation can cause microgliosis, inflammation, and establishment of thermal and mechanical hyperalgesia, its downregulation has been found to ameliorate the pain condition in both neuropathic and cancer pain [146–148]. Even though it is not fully clear how EZH2 is regulated, researchers described the involvement of mammalian target of rapamycin (mTOR) pathway and autophagy as eventual target of EZH2 modulation [149]. Its involvement in pain regulation is so deep that EZH2 has been accounted as biomarker in the evaluation of analgesic strategies for neuropathic pain [150]. What happens to be clear is that EZH2 is rigorously regulated in both in expression and activity by non-coding RNAs such as miR-124-3p, lncenc1, and miR-378 [151–153]. Non-coding RNAs such as miRNAs, circular RNAs (circRNAs), and long non-coding RNAs (lncRNAs), are key regulators of many molecular mechanisms, also in pain context [154]. Regarding miRNAs, their profiles are altered in people experiencing pain showing impaired regulation of their downstream targets [155]. Physiologically, miRNAs are able to block mRNA translation by binding to 3' untranslated regions thereby promoting mRNA degradation [154]. The multiplicity of their targets reflects the complexity of the underlying molecular mechanisms: pain-related genes of signaling molecules, ion channels, transcription factors, and inflammatory mediators are under miRNAs regulations [156–158]. Starting from the regulatory activity of one miRNA, several downstream mRNAs can be modulated. An example of this can be miR-183 which regulates the expression of more than 80% of pain-related genes among which *TRPV1* [159]. Nonetheless, miRNAs are under the regulation of other non-coding RNAs such as circRNAs and lncRNAs via complementary base pairing. Evidence of their involvement in the regulation of pain-related genes such as *KCNN1*, *VEGFB*, and *G9A* have been found [160–162].

Overall, the epigenetic mechanisms involved in pain perception work together in a complex crosstalk. Indeed, non-coding RNAs directly suppress the production of pivotal enzymes while pain-related genes are under the regulation of DNA methylation and histone modifications [163,164]. Nonetheless, DNA methylation and histone

modifications can influence also the production of non-coding RNAs [158,165]. It will be crucial in the future to dissect the intricate mechanisms underlying this crosstalk to better understand the role of epigenetics in the pain context.

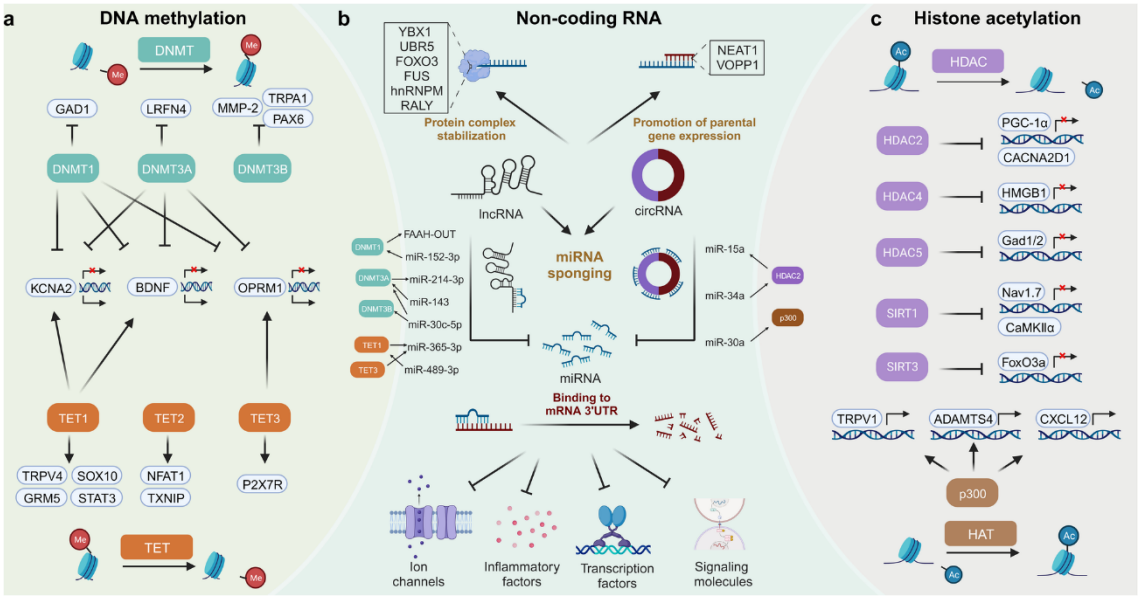


Figure 3: Epigenetic mechanism in pain regulation [15]. *A) DNA methylation; B) Non-coding RNA; C) Histone acetylation.*

2.3.3 PTMs

PTMs comprise a plethora of chemical modifications which regulate cellular localization, interactions, and activity of proteins. PTMs occur on specific groups in amino acids via, among all, ubiquitination, methylation, acetylation, phosphorylation, and glycosylation. In particular, around 600 PTMs have been identified so far, the most common of which are the ones previously cited [166]. In the context of pain, the most common PTM is undoubtedly phosphorylation. Indeed, the regulatory function of kinases is crucial in pain perception as phosphorylation involves VG ion channels and signaling pathways related to neuronal and non-neuronal functions and hyperalgesia. Few examples of the kinases involvement in pain perception are TRPA1 phosphorylated by cyclin-dependent kinases (CDKs), phosphorylation of Nav1.7 operated by protein kinase C (PKC) and Fyn, and phosphorylation of TRPM8 [167–169]. For instance, after nerve injury the establishment of inflammation can cause via BDNF as well as IL-33 phosphorylation and activation of Fyn, a member of the Src family kinase, inhibiting via

phosphorylation the NMDAR's subunit GluN2B, with increased NMDAR's membrane density and NMDAR-dependent synaptic current [170,171]. On the other hand, during acute and chronic pain decreased phosphorylation of TRPV1 can occur due to DRG-dependent production of programmed cell death ligand 1 (PDL1) [172,173]. PDL1 is responsible for attenuating VGSCs and enhancing VGPCs by phosphorylation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) and finally TRPV1 thereby causing the blockage of sensory neuron excitability [172,174]. Ubiquitination is a process of degradation of endogenous proteins performed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). It plays a role in pain mechanisms too. Indeed, the E3 Cbl Proto-Oncogene B (Cbl-b) in the spinal cord is responsible for processing the pain information and is dephosphorylated after noxious stimuli, reducing ubiquitination of the GluN2B subunit of NMDARs and tropomyosin receptor kinase A (TrkA) and promoting inflammatory pain [175,176]. Similarly, the E3 neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) stimulate pain hypersensitivity by promoting the targeted degradation of TRPA1, GluN2D subunit of NMDARs, and Nav1.7, among others [177–179]. The establishment of pain is characterized by epigenetic downregulation of NEDD4, suggesting the potential of NEDD4 as therapeutic target [178]. Moreover, the ubiquitin specific peptidase 5 (USP5), a deubiquitinating enzyme, can regulate ubiquitination and modulate VGCCs activity [180]. For instance, USP5 can be upregulated by IL-1β and TRPV1 causing and is able to directly interact with Cav3.2 enhancing cell ion currents and thereby stimulating neuropathic and inflammatory pain establishment [181,182]. Close to ubiquitination, sumoylation is pain-related PTM characterized by the covalent conjugation of small ubiquitin-related modifiers (SUMO) with target proteins influencing their localization, interaction and stability [183]. Sumoylation regulates ion channels in many ways. An example is represented by the sumoylation via SUMO-conjugating enzyme UBC9 of collapsin response mediator protein 2 (CRMP2), causing both enhancing membrane density and currents of Nav1.7 [184,185]. Interestingly, modified-CRMP2 is considered as marker of chronic neuropathic pain, rather than physiological pain, and it is under the regulation of other PTMs with painful and analgesic effects [186]. For instance, CDK5 can promote CRMP2 sumoylation via phosphorylation whereas Fyn-dependent phosphorylation downregulates it [187]. Nonetheless, antagonist of CRMP2 and UBC9 caused pain relief in trigeminal neuropathic pain [188]. Pain establishment is influenced by other PTMs as

well. Modulation of pain perception has been found for carbonylation, nitrosylation, succinylation, palmitoylation, and glycosylation [189–192]. The milder and reversible effects exerted by PTMs on proteins are overall a fundamental network underlaying pain establishment and modulation. Even though the current understanding about PTMs is limited, they represent an instrument of clinical value in the context of pain both in pain evaluation and treatment.

2.3.4 Microbiota

Microbiota is a symbiotic organism that comprise all the microbes which resides in the gastrointestinal and respiratory tracts, skin, and reproductive system, and is able to interact with the whole organism in which reside shaping both its physiology and pathology [193]. The regulation of pain perception is strictly influenced by microbiota via direct and indirect pathways (**Figure 4**). The direct pathways are a complex network in which microbiota-produced compounds play a crucial role. For instance, levels of histamine, which derives from decarboxylation of histidine, can cause abdominal pain via histamine receptor H1 activation and excitation of TRPV1 signaling [194]. The gut levels of histamine can raise due to the activity of gram-positive and gram-negative bacteria which exploit two different histidine decarboxylase, pyruvoyl-dependent and pyridoxal-dependent respectively [195]. In particular, the ectopic metabolism of histamine performed by *Klebsiella aerogenes* is the principal reason for abdominal pain during irritable bowel syndrome [196]. Regarding the indirect pathways, they involve the pathogen-associated molecular patterns (PAMPs), structural components of the microbiota such as lipopolysaccharide (LPS), flagellin, and teichoic acid, which are identified by host cells, that are responsible once recognized for the immune cells' activation and the release of chemokines and cytokines, but also neuropeptides, promoting a pro-inflammatory environment [197]. Thus, the immune system can identify microbiota dysbiosis via PAMPs resulting in inflammation and pain in a process mainly exacerbated by TNF-α [198]. Similarly, 5-HT is a by-product of *Streptococcus*, *Corynebacterium*, and *Enterococcus* that has been correlated with visceral hypersensitivity [199,200]. Indeed, the establishment of cannabinoid signaling due to time-dependent incremental interaction of 5-HT with receptors 5-HT3 and 5-HT4 cause exacerbation of pain hypersensitivity, which can be relieved via gut homeostasis modifiers to counter 5-HT levels [201,202]. Other metabolites involved in microbiota-

mediated shaping of pain perception are aspartate, glutamate, and GABA. For instance, *Bacilli* in the gut are able to produce excitatory neurotransmitters such as aspartate and glutamate, responsible for enhancing the sensory neurons excitability [203]. On the other hand, different bacteria are able to produce the inhibitory neurotransmitter GABA, such as *Bacteroides*, *Bifidobacteria*, and *Lactobacilli*, which can be modulated by administration of gut probiotics to effectively ameliorate visceral pain [204]. Interestingly, microbiota can also directly interact with neurons to produce pain without relying on the immune cells activation of released metabolites but exploiting toll-like receptors to detect eventual microbiota invasion. By detecting microbes' antigenicity, TLRs identify PAMPs and start the immune response and the downstream signaling pathways [205]. An example of this mechanism is the activation of TLR4, which is deeply involved in chronic pain establishment consistent with its expression on DRG and afferent fibers of the trigeminal nerves [206]. Under bacterial dysbiosis, TLR4 can be activated by LPS causing increased firing rates of neurons expressing TRPV1, leading to pain hypersensitivity [207]. Notably, these events can be observed both in CNS and PNS. Indeed, TLR4 is upregulated in the hippocampus and the prefrontal cortex during visceral pain, suggesting the relevant role of the gut-brain axis in pain perception [208]. Similarly, TLR5 can interact with flagellated bacteria to promote chronic abdominal pain, as observed in mice [209]. Conversely, analgesic effects via TLR5 have been observed too [210]. Flagellin combined with the membrane-impermeable lidocaine derivative QX-314 caused a TLR5-dependent inhibition of sodium currents, predominantly in A-type fibers in mice. The interaction flagellin-TLR5 responsible for QX-314 entrance in neurons permitter the alleviation of mechanical allodynia induced by nerve injury, chemotherapy, and diabetic neuropathy in mice [210]. Overall, microbiota is crucial in influencing pain perception both centrally and peripherally. Distinct microbial patterns linked to pain have been identified. The pathways mediating the reciprocal communication between the microbiota and the nervous system are day-by-day becoming clear, opening new avenues for clinical advancements in pain management.

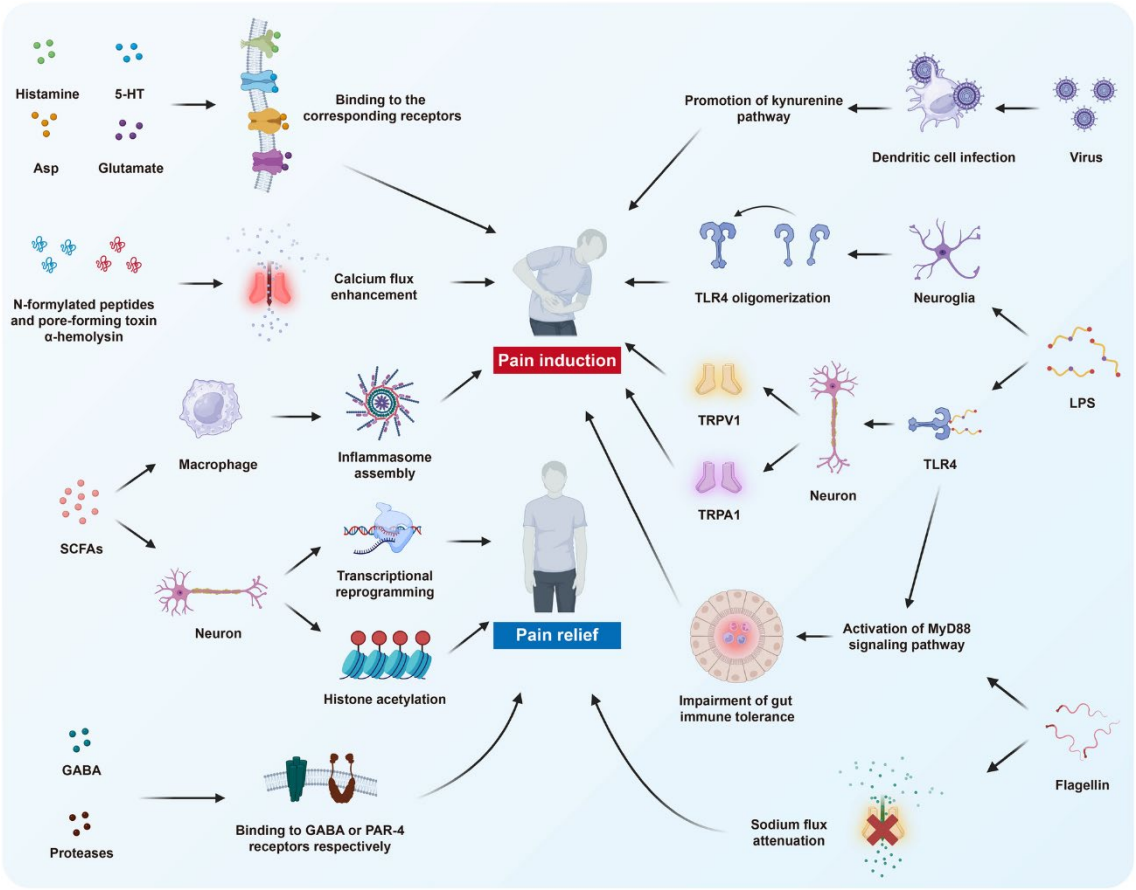


Figure 4: Microbiota involvement in pain modulation [15].

2.4 Pathways involved in pain modulation

Signaling pathways are cascade of biochemical events within cells activated by distinct stimuli. Physiological and pathological processes are shaped by these pathways, influencing gene regulation and cellular functions. Emerging evidence has revealed complex links between altered signaling networks and pain perception. Moreover, preclinical and clinical studies highlighted the therapeutic potential of targeting disrupted signaling pathways to mitigate pain. Among the most critical pathways involved in pain perception there is the mitogen-activated protein kinase (MAPK) pathway. MAPK signaling comprise several branches including p38, extracellular signal-regulated kinases (ERKs), and C-Jun N-terminal kinase (JNK), all involved in the modulation of proliferation, differentiation, but also inflammation [211]. During acute pain, MAPK pathway is activated via TNF-α causing a p38-mediated hyperphosphorylation of the ser110 of Nav1.7, leading to increased membrane insertion of the channel [212]. Other VGSCs are influenced by these events. For instance, Nav1.8 and Nav1.9 are upregulated

under these conditions, supporting the formation of pain hypersensitivity [213]. Moreover, evidence of upregulation of Nav1.6 during CIPN via MAPK/ERK signaling have been found as well [211]. MAPK signaling activation via p38 can occur under NGF action with upregulation of acid-sensing ion channel 3 (ASIC3), a neuronal voltage-insensitive sodium channels, in DRG and promotion of mechanical allodynia [214]. On the other hand, inhibition of peripheral NGF derived from Schwann cell and macrophages ameliorated neuropathic pain via inhibition of transforming growth factor-β-activated kinase 1 (TAK1)-dependent MAPK signaling in DRG and sciatic nerve of rats with chronic constriction injury-related pain [215]. MAPK signaling is an intricate network of events in which different regulation of its branches bring to different downstream effects. Indeed, even though MAPK signaling can promote pain hypersensitivity, opposite effects have been described in which p38 upregulate the spinal glial transporter of glutamate averting the long-lasting non-evoked resting pain, as described in rats with surgical incision [216]. Overall, MAPK signaling is pivotal in pain regulation and several strategies are under active research to interfere with its modulation to shape a better condition in patients with pain [217–221]. However, other pathways are involved. Among the most critical pathways the Wnt/β-catenin one is crucial for many physiological processes, morphogenesis, and oncogenesis. Once Wnt is activated, the β-catenin destruction complex cannot form, and the complex is translocated to the nucleus where it can regulate the gene expression [222]. Wnt involvement with pain regulation has been observed, for example, in neuropathic pain *in vivo* models in which upregulation of Wnt3a, Wnt5a, and Wnt10a can be found [223,224]. Wnt/b-catenin signaling is relevant for intercellular communication during neuropathic pain. Moreover, secretion of Wnt5 from A-type fibers in DRG can promote fast currents of nociceptive neurons expressing TRPV1. GPCRs such as GPR177 can be responsible for Wnt secretion [225]. Nonetheless, GPR177 is not the only regulator of this pathway: lysophosphatidic acid receptor 3 (LPAR3), secreted frizzled-related protein 1 (SFRP1), long non-coding RNA-CRNDE and miR-26a-5p have control over this pathway and thereby on pain regulation [224,226–228]. Wnt/β-catenin signaling can promote inflammatory pain by stimulating production and release of TNF-α, IL-18, and substance P, but also potentiate neuropathic pain via upregulation of the vesicular glutamate transporter 2 (VGLUT2), a critical component of glutamate transmitting system [229–231]. Moreover, activation of this pathway in primary sensory neurons can lead a calcium-dependent translocation to the membrane of P2X3 and GluN2B causing

peripheral hypersensitivity [232]. Regarding AMP-activated protein kinase (AMPK) signaling, its metabolism-related activity modulates pain perception in different ways [233]. For instance, AMPK is an intracellular glucose sensor whose signaling is involved in hyperalgesia [234]. DRG neurons exposed to high glucose levels show inhibition of AMPK signaling, with increased expression and activity of TRPA1, leading to the TRPA1-dependent hyperalgesia characteristic of the diabetic neuropathy [234]. Activation of AMPK signaling can also bring to analgesia via polarization of anti-inflammatory microglia or by modulating the endocannabinoid metabolism of macrophages [235,236]. Moreover, AMPK signaling is able to intensify the autophagic activity in Schwann cells promoting cell clearance and thereby ameliorating the peripheral neuropathy [237]. Analgesic effects related to autophagy can also be obtained via modulation of phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin pathway. Indeed, the release of BDNF from bone marrow mesenchymal stem cells during exercise in mice with sciatic nerve injury promoted the transition from pro-inflammatory to anti-inflammatory microglia via modulation of the PI3K/Akt/mTOR pathway with enhanced autophagy [238]. However, pro-inflammatory microglia-dependent downregulation of this pathway can cause astrogliosis promoting chronic pain [239]. Thus, PI3K/Akt/mTOR pathway shows both beneficial and detrimental effects, eventually depending on specific cell type and pain condition. Indeed, PI3K/Akt/mTOR pathway can also alleviate neuropathic pain via nerve regeneration after nerve injury [240]. Moreover, this pathway can be involved in other cell functions other than autophagy such as cell survival, angiogenesis, proliferation, and metabolism. Overall, PI3K/Akt/mTOR pathway hyperactivation is linked to nociception and neuropathic pain [241,242]. For instance, Akt can be responsible for ASIC1a phosphorylation causing increased surface expression and activity of ASIC1a, resulting in pain hypersensitization [243]. Again, activation of mTOR complex 2 has been related to increased structural plasticity of nociceptive ends leading to inflammatory pain [241]. Interestingly, PI3K/Akt/mTOR pathway can be regulated by glia-derived neurotrophic factor (GDNF) secreted by bone marrow mesenchymal stem cells. Such mechanism appears to be related in rats to glial polarization with upregulation of PI3K/Akt signaling and downregulation of the nuclear factor kappa B (NFkB) pathway, thereby alleviating abnormal discharge of C-type fibers [244]. In the regard of NFkB signaling, such pathway is upregulated in DRG and spinal cord of animals with neuropathic pain and it is strictly associated with inflammatory events in CNS, ending in further promotion of neuropathic pain [245].

NFkB is a complex composed as hetero- or homodimers of the Rel proteins, among which RelB, c-Rel, p65, p50, and p52 [246]. Usually, the heterodimer p65/p50, the NFkB inactive form, is fixed in the cytoplasm due to inhibitor of kB α (IkBα). However, pro-inflammatory molecules and growth factors can stimulate the phosphorylation of IkBα by IkB kinase, causing its degradation thereby releasing p65/p50 [247]. The free NFkB heterodimer translocate to the nucleus causing the expression of inflammatory and pain-related proteins, among which IL-1β, TNF-α, and cyclooxygenase 2 (COX-2) [246,248]. Interestingly, these pathways are deeply interconnected. For instance, TNF-α can activate p38, MAPK, and NFkB to initiate pain as mentioned above, but it can also activate JNK to maintain pain condition [249]. Moreover, establishment of pain can cause the activation of c-Fos and JUN, key markers of neuronal activation and pain development [250]. Indeed, the combination of c-Fos and JUN produce the complex activation protein 1 (AP1) that translocate to the nucleus acting as transcription factor for genes of NGF, neuropeptides, and opioid peptides, all relevant in the pain condition [251,252]. Similarly, IL-6 induces activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway which is crucial in pain maintenance [253]. The IL-6 binding with the IL-6 receptor causes its dimerization and the autophosphorylation of two JAK proteins among JAK1, JAK2, JAK3, and TYK2 and the following docking of STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A/B, and STAT6). Then JAK-mediated phosphorylation of the STAT proteins occurs, causing the separation of the phosphorylated STAT from the receptor and its translocation to the nucleus in which will regulate the expression of many genes [254]. In particular, IL-6-mediated phosphorylation of JAK1 and JAK2 and the resulting phosphorylation of STAT3 is a key event for the establishment of chronic inflammation and pain [255]. Therefore, signaling pathways exhibit intricate interconnections. A single molecular target or pain characteristic can be influenced by multiple pathways. This intertwined network plays a key role in both the amplification of pain sensitivity and its amelioration. Similarly, disrupting critical points within these pathways might lead to unforeseen adverse effects, potentially hindering the clinical development of pathway-targeting agonists or inhibitors. More rigorous investigations are needed to thoroughly evaluate their efficacy and safety.

2.5 Categories of pain

Starting from its fundamental causes, different types of pain can be identified: nociceptive, nociplastic, and neuropathic pain. Regarding nociceptive pain, it refers to physiological pain as defensive system against noxious stimuli, for instance inflammatory pain. It is a transient event naturally occurring in response to both real and potentially harmful stimuli, causing protective actions. Nociceptive pain appears as a blur sensation when it is a visceral stimulus while somatic pain can be burning and sharp and it is usually sensed at the dermis layer [256]. On the other hand, nociplastic pain is characterized by the lack of a clear activation of the nociception with, nonetheless, clinical evidence of altered nociception. Interestingly, this type of pain does not match with both nociceptive pain and neuropathy given the absence of an organic lesion coexisting with a distinguished pain phenotype [257]. Environmental, psychological, and genetic factors contribute to the establishment of this kind of pain in different ways, making it possible to distinguish between different categories of nociplastic pain: chronic primary musculoskeletal pain, chronic primary headache pain, chronic widespread pain, complex regional pain syndrome, and chronic primary visceral pain [258]. The etiology of this type of pain is challenging as it comprise numerous factors among which organ damage, nerve invasion, and immunological impairment [259].

Regarding neuropathic pain, the following chapters will dissect the main features of this pain typology with a peculiar focus on CIPN and its underlying mechanisms.

CHAPTER III

3.1 Neuropathic pain: definition and epidemiology

As stated by IASP, neuropathic pain is a painful state caused by lesions or diseases which affects both the central neurons and the peripheral fibers of the somatosensory nervous system [260]. Indeed, neuropathic pain is a secondary chronic condition that arise as maladaptive response of the nervous system to "damage" in cancer (e.g., CIPN) but also following diabetes (e.g. peripheral diabetic neuropathy), autoimmune disorders affecting the CNS (e.g., multiple sclerosis), viral infections (e.g., herpes zoster), and traumatic damage to the nervous system (e.g. spinal cord injury) and so on [261]. The outcomes of the nerve injury are various and the underlying mechanisms change even within the same condition, with patients' differences in both symptoms and treatment effects [262]. Patients with such distressful conditions can usually experience shooting pain, spontaneous or evoked pain, and tingling following either harmful or non-harmful stimuli, potentially reaching disability over time with loss of life's quality and increased healthcare cost [263]. Moreover, neuropathic pain shows considerable difficulties in therapeutic management, as only a minority of patients achieve substantial relief from a given therapy, with weak response to analgesic drugs thereby causing psychological features like anxiety, insomnia, and depression [261,264]. Even though prevalence and incidence of neuropathic pain are hard to estimate because of the absence of consensus on its definition, epidemiological studies showed that 7 to 10% of the global population run into neuropathic pain, with increased prevalence among women (60,5% of all patients) and overall, in people with chronic diseases. Indeed, of all the patients with chronic pain, the 20-25% of them experience neuropathic pain [265,266]. Known as one of the most challenging areas in healthcare, neuropathic pain remains difficult to manage, even with recent advancements. However, there have been notable improvements in understanding its underlying mechanisms, leading to new developments in both medical and surgical treatments [267]. Although the various therapies work through different mechanisms, they all aim to modify neural pathways, adjust neuron activity, and improve communication between nerve cells. There is a critical need to deepen the knowledge of these pathological mechanisms to enable the creation of more precise therapies.

3.2 Clinical perspective

Symptoms of neuropathic pain are strictly dependent on cause and location of the nerve injury [268]. Overall, patients can experience both sensory gain and loss. Indeed, they can perceive pain after non-painful stimuli (allodynia) and pain perception can also be boosted by neuropathic pain after painful stimuli (hyperalgesia) experiencing more pain than what should be perceived. Such pain can also be perceived as persistent or intermittent spontaneous pain with odd sensations resembling itching, needle bites, tingling, and reduced sensitivity or sensory loss [269]. Neuropathic pain greatly burden the life of patients, reducing their quality of life at both the physical and emotive levels. Moreover, pain can be refractory to therapies making it difficult to handle and leading patients to developing depression, anxiety, and other psychological impairments as mentioned above [270]. Furthermore, misdiagnosis is a common problem caused by difficulties in recognizing neuropathic pain, suggesting the need for a more in depth evaluation of the patient's clinical condition [271]. Indeed, it is crucial to perform a comprehensive physical and neurological exam, focusing on when the pain started, where it is located, how it spreads, and whether it is linked to injury or sensory alterations. Rating scales, such as the numeric rating scale and the visual analogue scale, and questionnaires are useful instruments for evaluation of neuropathic pain symptoms and intensity [272]. Diagnostic investigations to establish the existence of provable lesions to the somatosensory system can be exploited: few example are quantitative sensory testing, neurophysiological testing, and skin biopsy [273]. Regarding quantitative sensory testing, it evaluate nociceptive and non-nociceptive pathways by exploiting thermal and mechanical stimulation both peripherally and centrally, helping in the detection of sensory loss as well as paresthesia, hyperalgesia, and allodynia. In order to address the lesion, its entity and location, neurophysiological tests like evoked potentials are performed. Contact or radial thermal stimulation can give rise to evoked potentials via contact or laser heat allowing the demonstration and quantification of lesions along the central and peripheral pathways. However, both quantitative sensory testing and neurophysiological testing can be not sufficient for a proper diagnosis. In such cases and in the suspicion of small fiber neuropathy it is possible to perform skin biopsy, a non-invasive technique by which it is possible to collect intraepidermal C-type fibers. Their histological analysis will potentially provide confirmation of a somatosensory lesion [274]. Even though clinical evaluation and objective diagnostic tools can offer a picture of the patients in some cases, other people would require history

analysis to define the probable pain condition, highlighting the pivotal role of the complex clinical judgement of the physicians [272].

3.3 Pathophysiology of neuropathic pain

Once a peripheral nerve lesion take place, inflammation spreads locally and mast cells and macrophages, as resident immune cells, and Schwann cells react to the traumatic event [275]. Indeed, a complex but well-orchestrated cycle of events starts at the injury site, with Schwann cells differentiating to proceed with the removal of the myelin sheath and mast cells recruiting neutrophils (**Figure 5**). In particular, degranulating mast cells release pro-inflammatory molecules, among which growth factors, histamine, leukotrienes, and serotonin that contribute to nociceptors sensitization and neutrophils infiltration. The latter, similarly, release pro-inflammatory molecules that promote the local recruitment of T cells and other macrophages and the sensitization of nociceptors as well. On the injury site, macrophages promote inflammation by releasing pro-inflammatory cytokines and chemokines and participate in the degradation via phagocytosis of the lesioned myelin sheaths and axons with Schwann cells. Also, nociceptors can be upregulated in some measure after lesions and T-helper cells, by releasing both pro- (e.g., IL-1β, IL-17, and TNF-α) and anti-inflammatory (e.g., IL-4 and IL-10) mediators at the injury site, can directly modulate their activity [275]. However, these events are not limited to the periphery. For instance, peripheral lesions can cause disruption of the blood-nerve barrier at the spinal cord level causing influx from periphery of immune cells [276]. Thus, inflammation reaches CNS from periphery stimulating, here, glial cells such as microglia and astrocytes which play a pivotal role in the establishment and the maintenance of a pro-inflammatory environment thereby promoting nociceptors hypersensitivity and modulation of sensory neuron gene expression [239].

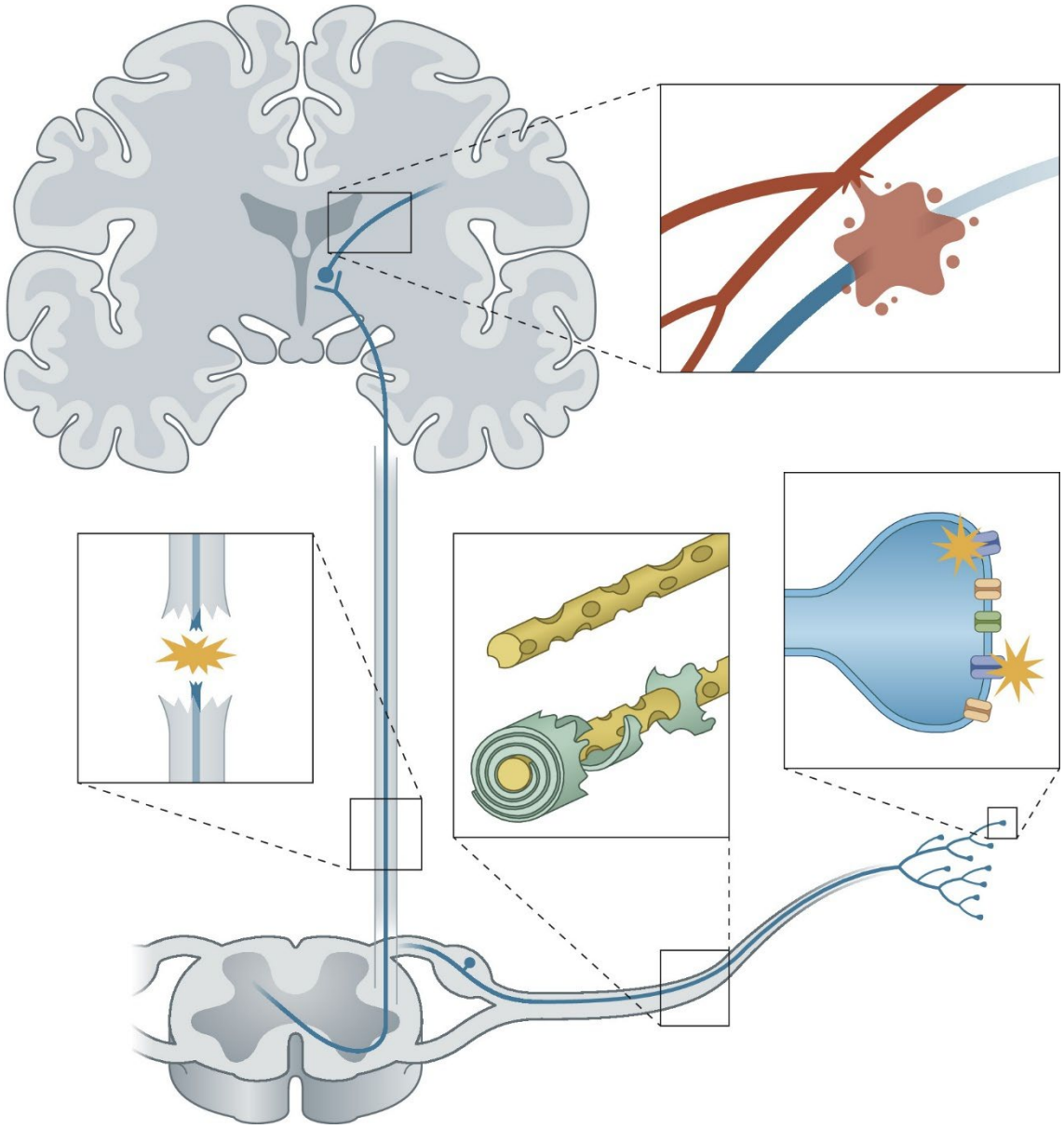


Figure 5: Degenerative mechanisms underlying neuropathic pain [277].

3.3.1 Peripheral neuropathic pain

As mentioned above, lesions to peripheral nerves can lead to neuropathic pain development. Even though the link is clear, there is a great gap between lesions' type (e.g., crushing, transecting, stretching) and severity, and neuropathic pain development. Indeed, the establishment of pain does not characterize every patient with nerve damage and the underlying mechanisms probably are an interplay of peripheral and central changes, with psychological and genetic factors [278,279]. The most involved fibers in the establishment of neuropathic pain are types C and Aδ, which can react to various

stimuli such as thermal, mechanical, and even chemical. In patients with neuropathic pain, non-lesioned nociceptive afferents placed near to the injured ones shows ectopic nerve activity, as described via microneurography, which is attributed to channelopathy [280]. By lowering the threshold of action potential and increasing membrane excitability the channelopathy causes spontaneous ectopic nerve activity. In the end, the subject can experience paroxysmal lancinating pain or tingling without any kind of damage [275,281].

3.3.2 Central neuropathic pain

Once damage is caused by lesions or diseases to the central somatosensory system, neuropathic pain occurs due to central sensitization immediately following the insult or within one year, with some patients finding it resolved after around 12 months and others developing chronic pain [277]. Central neuropathic pain is a common condition in people with spinal cord injury (~50% of patients), but also in people with multiple sclerosis (20%) and stroke (8-10%) [282–284]. Main symptoms are hyperalgesia, ongoing summation, allodynia, and aftersensation [285]. Other than CNS' lesions, the central sensitization can also occur due to ectopic activity in afferent fibers and loss in the superficial dorsal horn of the spinal cord of the inhibitory GABAergic interneurons. Overall, these events are responsible for impeding analgesic descending pathways which originate in the brainstem. In the end, there is no inhibition for painful pathways, with pain-related symptoms caused not only by the injured nerve but also by the activity of intact ones [280]. Indeed, the loss of GABAergic inhibition within the dorsal horn is selective and does not involve, for example, the excitatory molecules which will continue to exert their activities among which VGSCs overexpression [286]. Over time, changes at the postsynaptic level in second-order neurons will support the maintenance of this hyperexcitability which, with overexpression of low-threshold mechanosensitive Aβ-type and Aδ-type afferent fibers, will result in establishment of the symptoms state above [280,286,287].

3.4 Overview on peripheral neuropathies

Peripheral neuropathies show from diverse origins, including genetic mutations, physical trauma, exposure to toxins, or inflammatory damage to peripheral nerves [288]. As some of the most widespread neurological disorders, they are estimated to affect 77 out of 100,000 individuals annually, with prevalence rates ranging from 1–12% across all ages, reaching 30% in elder people [289]. The wide variety of neuropathies exceed 100 distinct types with common symptoms including sensory disturbances such as numbness, pain, weakness, and altered sensations, each tied to specific nerves involvement complicating accurate diagnosis and treatment strategies [290]. For instance, conditions like diabetic and chemotherapy-induced neuropathies may present as either painful or painless, underscoring the intricacy of their clinical profiles. Painful neuropathies can be divided into two broad categories: hereditary and acquired. Advances in next-generation sequencing (NGS) have unveiled genetic factors linked to hereditary forms. For instance, VGSCs play critical roles in neuropathic pain, as mentioned in the previous chapters. Gain-of-function mutations in the SCN9A gene results in heightened pain sensitivity in conditions like erythromelalgia and paroxysmal extreme pain disorder [291,292]. These mutations amplify sodium influx, driving hyperexcitability of nociceptors [290]. Acquired neuropathies are predominantly linked to systemic diseases like diabetes, alcohol abuse, or toxic agents, including chemotherapy. A notable example is small fiber neuropathy, characterized by degeneration of epidermal nerve endings and reduced intraepidermal fiber density, often observed in diabetic patients [293]. Small fiber neuropathy symptoms, such as hyperalgesia and allodynia, correlate with neuronal hyperexcitability driven by altered calcium levels and aberrant activity in DRG neurons [294]. Regarding CIPN, a prevalent side effect of cancer treatments, manifests as pain and sensory deficits in a "glove-and-stocking" pattern, profoundly impairing patients' quality of life [295].

CHAPTER IV

4.1 CIPN

CIPN is a prevalent and incapacitating side effect associated with cancer treatments. This condition impacts almost the 90% of individuals undergoing therapies involving taxanes like paclitaxel and docetaxel, vinca alkaloids such as vincristine, platinum-based compounds like oxaliplatin and carboplatin, or proteasome inhibitors like bortezomib [296,297]. The clinical manifestations of CIPN are diverse, spanning both sensory and motor domains. Common symptoms include sensations of tingling, numbness, burning or shooting pain, impaired proprioception, and diminished tactile sensitivity. Motor dysfunctions such as muscle weakness, coordination problems, and difficulty with fine motor tasks also occur frequently [298]. In clinical practice, CIPN management often necessitates modifications to chemotherapy regimens, including dose adjustments, temporary pauses, or complete discontinuation of treatment [299]. However, such measures can undermine therapeutic efficacy potentially affecting survival outcomes [300]. The management of various malignancies, including breast, prostate, and colorectal cancers, alongside gynecological tumors, testicular cancer, and hematological cancers like multiple myeloma, heavily relies on these drugs [297]. Therefore, CIPN is a frequent challenge in oncology care. CIPN frequently exerts a long-term impact on patients' lives. Chronic and persistent symptoms are reported by up to 42% of individuals, persisting long after cessation of the causative agents [301]. Such condition is linked to a significant decline in quality of life and is accompanied by psychological and behavioral challenges across the cancer care continuum [295,302]. Patients have highlighted the burden of CIPN on their daily routines, describing difficulties in performing tasks such as fastening buttons, operating electronic devices, or maintaining mobility [303]. Moreover, the limitations imposed by CIPN extend beyond physical discomfort, often leading to a sense of frustration and reduced independence. These challenges emphasize the need for effective preventive and therapeutic strategies to address CIPN. Current management options remain inadequate for mitigating its debilitating effects, underscoring the importance of interventions that alleviate symptoms while preserving the integrity of chemotherapy regimens. Further research into such approaches is critical to improving outcomes and enhancing the quality of life for patients experiencing CIPN.

4.2 Molecular mechanisms underlying the establishment of CIPN

CIPN remains a significant complication in oncology, with a complex and multifactorial pathogenesis (**Figure 6**). This section focuses on the principal molecular and cellular mechanisms implicated in its development.

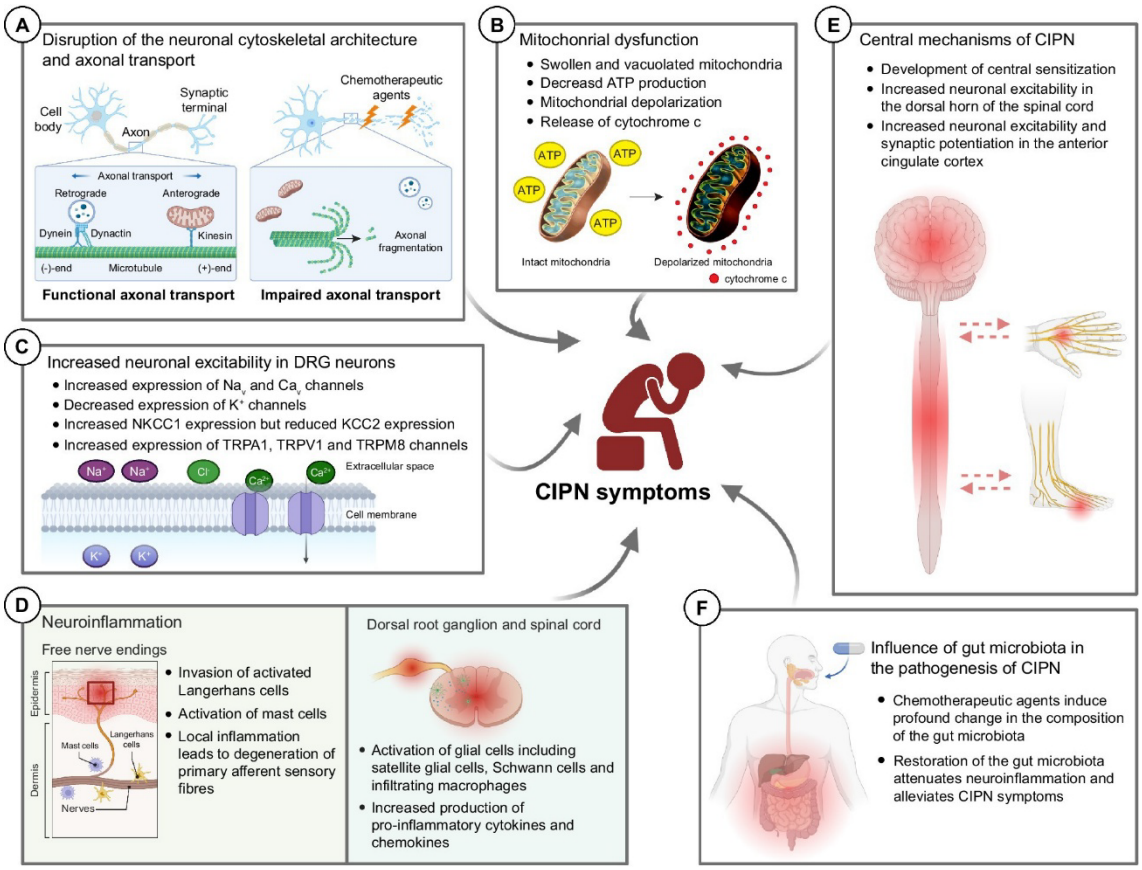


Figure 6: Overview on the molecular mechanisms underlying the establishment of CIPN [304]. *A) Disruption of the neuronal cytoskeleton; B) Mitochondrial impairment; C) Hyperexcitability in DRG neurons; D) Neuroinflammation; E) Central events of CIPN; F) Influence of gut microbiota in CIPN.*

4.2.1 Disruption of the neuronal cytoskeleton

Chemotherapeutics like vincristine and paclitaxel exert their anticancer activity by modulating microtubule dynamics: vincristine inhibits microtubule polymerization while paclitaxel promotes it [305,306]. These mechanisms result in cell cycle arrest and apoptosis in cancer cells [304]. However, chemotherapeutic drugs can cross the blood-

nerve barrier and bind to β-tubulin, which is critical for neuronal cytoskeleton stability, particularly in peripheral nerve fibers and sensory neurons [307]. Their interaction disrupts the cytoskeletal structure of healthy peripheral neurons, such as DRG cells, in cancer patients significantly altered the mechanical properties of DRG neurons due to excessive microtubule depolymerization (vincristine) or polymerization (paclitaxel). Moreover, confocal imaging demonstrated that vincristine and paclitaxel caused cytoskeletal disorganization leading to impaired neurite outgrowth [308,309]. Similarly, bortezomib, which is a proteasome inhibitor that enhance microtubule polymerization in neuronal cells, causes excessive stabilization of microtubules and axon fragmentation by increasing hyper-stable delta 2 tubulin (D2) levels in DRG neurons, with impaired neurite outgrowth as well [310]. Substantial evidence indicates that vincristine, paclitaxel, and bortezomib promote axonal degeneration and the loss of axonal remodeling capacity appears to drive degeneration of distal sensory nerve terminals, leading to altered pain perception and hypersensitivity [311,312]. Interestingly, the integrity of the microtubule cytoskeleton is essential for axonal transport of organelles such as mitochondria and lysosomes, but also mRNA, vesicles and other factors [313]. Impaired axonal transport is a key contributor to axonal degeneration in peripheral neurons in CIPN, suggesting the therapeutic relevance of impaired axonal transport as therapeutic target in this context [314].

4.2.2 Mitochondrial impairment

Mitochondria play a critical role in regulating intracellular calcium levels under physiological conditions [315,316]. Neurons are particularly susceptible to mitochondrial impairment due to their high energy demands, which are necessary for maintaining resting membrane potentials, conducting and propagating electrical signals, and facilitating pre- and post-synaptic neurotransmission [316]. Given their highly polarized structure, neurons heavily rely on efficient anterograde and retrograde mitochondrial transport to sustain energy homeostasis [317–319]. Indeed, disruptions in mitochondrial ATP exchange or transport can precipitate energy deficits and axonal degeneration in these cells [316,319]. Interestingly, mitochondrial impairment and impaired mitochondrial transport are involved in the pathogenesis of CIPN [320]. Platinum-based chemotherapeutic agents like cisplatin and oxaliplatin exert their anti-cancer effects by forming DNA-platinum adducts, which disrupt DNA replication in

malignant cells, thereby inducing apoptosis [321]. Unfortunately, cisplatin's off-target effects extend to peripheral neurons, where it binds to mitochondrial DNA in DRG cells disrupting mitochondrial DNA replication and transcription, leading to mitochondrial dysfunction. Moreover, due to the absence of DNA repair machinery in mitochondria, damage inflicted by cisplatin remains unrepaired, triggering cytochrome c release and apoptosis in DRG neurons [322]. Paclitaxel affects mitochondrial function by binding to β-tubulin in the mitochondrial membrane, causing the opening of the mitochondrial permeability transition pore, leading to rapid mitochondrial depolarization, cytochrome c release, and reduced ATP production in neuroblastoma cells and DRG neurons [323]. Similarly, in animal models of bortezomib-induced neuropathy, peripheral nerves exhibited mitochondrial swelling and vacuolation in both myelinated A-type fibers and unmyelinated C-type fibers [324]. These structural mitochondrial abnormalities severely impaired ATP production, highlighting bortezomib's mitotoxic effects. Finally, vincristine, for instance, causes rapid mitochondrial depolarization at distal axonal ends, leading to mitochondrial swelling and demyelination in peripheral nerves [325]. In conclusion, preserving mitochondrial integrity and functionality is vital for preventing CIPN. Therapeutic strategies enhancing mitochondrial ability to counter the described toxicity could offer significant benefits in mitigating neuronal damage and improving patient outcomes in CIPN management.

4.2.3 Hyperexcitability in DRG neurons

The somatic pain detection involves free nerve endings of musculoskeletal afferents, with nociceptive ion channels at the nerve terminals activated by specific stimuli to transduce pain signals [326]. Transcriptomic shifts induced by chemotherapy in the cell bodies of DRG neurons, as revealed through RNA sequencing, have been shown to potentially increase neuronal excitability and contribute to persistent pain [296]. For example, lumbar DRGs exhibit upregulation of GPCRs and ion channels in response to paclitaxel, linking these changes to neuropathic pain [327]. Similarly, heightened expression of Nav, Kv, and TRP channels has been observed in the DRGs and spinal dorsal horns of paclitaxel-treated rodents [328]. Studies have also demonstrated that paclitaxel triggers the activation of the mTOR and MAPK-interacting kinase (MNK)-eukaryotic initiation factor 4E (eIF4E) pathways, altering the translation of GPCRs and ion channels in DRG neurons [329]. Pharmacological inhibition of this pathway through

eFT508 has been effective in reversing paclitaxel-induced hypersensitivity. Oxaliplatin has been shown to alter the expression of ion channels and synaptic genes in rat DRGs [330]. Functionally, ion channels play a key role in the pathogenesis of CIPN. For instance, paclitaxel-induced upregulation of Nav1.7 in DRG neurons and dorsal horn nerve terminals contributes to spontaneous ectopic activity and targeting Nav1.7 with selective blockers like ProTx II has been found to alleviate hyperalgesia [331]. The trafficking of Nav1.7 vesicles to nerve endings is also enhanced by paclitaxel, leading to increased channel expression [332]. Similarly, vincristine has been shown to elevate Nav1.7 and Nav1.8 expression in DRG neurons and spinal cords, with pharmacological inhibition or gene silencing of Nav1.7 reducing neuronal hyperexcitability and mechanical allodynia [333,334]. Furthermore, paclitaxel promotes Nav1.8 transport to distal axons, increasing its surface expression [335]. In the case of oxaliplatin, Nav1.6 expression in DRGs is upregulated, which induces cold allodynia. Both knockout models and specific antagonists for Nav1.6 have been shown to reverse oxaliplatin-induced allodynia, underscoring its importance [336,337]. Regarding potassium channels, reduced Kv subunit expression in DRG neurons following injury has been linked to neuropathic pain [338]. Deficiencies in Kv1.1 or Kv1.2 can cause mechanical and cold allodynia, as observed in mice [339]. K2P channels, which help maintain resting membrane potential, are significantly downregulated in DRGs under pathological conditions, contributing to hypersensitivity [340]. In DRGs exposed to oxaliplatin, reduced expression of Kv1.1 and K2P channels such as TREK1 and TRAAK has been observed, and neuropathy-like symptoms are replicated when these channels are co-ablated [341]. Paclitaxel has also been reported to reduce potassium channel expression, including KIr1.1, KIr3.4, and K2P1.1, although the underlying mechanisms remain unclear [342]. Hyperexcitability induced by paclitaxel also affects KCNQ channels, but Kv7 channel openers like retigabine can reverse this effect [343]. Calcium influx through VGCCs is essential for processes such as neurotransmitter release, gene activation, and muscle contraction as previously described [20]. Neuropathic pain models have demonstrated the efficacy of gabapentin and pregabalin, which target the Cavα2δ1 subunit, in reducing mechanical and thermal hyperalgesia [344]. Elevated expression of α2δ1 in DRGs after treatment with paclitaxel or oxaliplatin underscores its role in CIPN, and gabapentin has shown therapeutic promise in mitigating these symptoms [345]. TRP channels, which detect noxious stimuli, are emerging as potential analgesic targets. TRPA1 and TRPV1, critical for thermal and chemical sensation, are upregulated in

DRGs after chemotherapy. Genetic or pharmacological inhibition of these channels has been effective in alleviating hypersensitivity [346,347]. TRPM8, associated with cold sensitivity, also undergoes alterations, although its role in CIPN requires further exploration [348]. Despite significant advancements in understanding ion channel modulation in CIPN, identifying upstream regulatory mechanisms and novel therapeutic targets remains critical for developing more effective treatments.

4.2.4 Central events of CIPN

As previously discussed, central sensitization is a key factor in the onset of chronic pain, including CIPN, with central mechanisms playing a more prominent role than peripheral ones. While peripheral sensitization involves nociceptor hyperactivity at axonal terminals, central sensitization is characterized by increased excitability of nociceptive neurons in the spinal dorsal horn and CNS [277]. This leads to amplified responses to noxious and non-noxious stimuli, expanded receptive fields, and hypersensitivity to stimuli [280]. Sustained activation of C-fiber nociceptors by intense stimuli causes glutamate release, which binds to NMDA receptors in the spinal dorsal horn, initiating central sensitization [304]. Blocking NMDA receptor activity, via MK801 or D-CPP, or deleting the NR1 subunit effectively reverses this sensitization [349]. These findings underscore NMDA receptors' significance in central sensitization and suggest their therapeutic potential for chronic pain [350]. For instance, oxaliplatin at trace levels in cerebrospinal fluid caused dorsal horn hyperexcitability and mechanical allodynia in rats [351]. Elevated C-X-C motif chemokine (CXC) ligand 3 (CXCL3) levels in the spinal cord partially explained this effect, as neutralizing CXCL3 eliminated neuronal hyperactivity. Moreover, these agents induced astro- and microgliosis, triggering inflammation in the dorsal horn. Cytokines such as TNF-α, IL-1β, and IL-6, alongside neuropeptide substance P, contribute to neuronal hyperexcitability [352,353]. Reducing microglial P2X7 receptor activity or administering minocycline alleviates inflammatory mediator production and chronic pain [354]. Neurons in the spinal dorsal horn relay sensory input to the thalamus, which processes pain signals through the amygdala and anterior cingulate cortex [355]. Chronic pain enhances excitability in glutamatergic pyramidal neurons of the anterior cingulate cortex, reduces GABA availability, and alters synaptic plasticity, as seen with paclitaxel treatment [356]. Targeting anterior cingulate

cortex neuronal plasticity, including glutamatergic inhibition or GABA supplementation, is promising for CIPN therapy [357].

4.2.5 Influence of gut microbiota in CIPN

Recent studies increasingly highlight the involvement of gut microbiota in pain-related pathologies [358,359]. In murine models, significant alterations in microbiota composition were detected after spared nerve injury, with an increase in *Staphylococcus sp.* coinciding with severe mechanical allodynia [360]. Similarly, chronic constriction injury promoted the prevalence of *Streptococcus*, *Lactobacillus*, *Helicobacter*, *Blautia*, *Christensenella*, and *Phascolarctobacterium*, with notable changes in serum metabolite profiles. Network analyses linked these microbiota shifts to impaired lipid metabolism, altered molecular transport, and heightened inflammation in chronic constriction injury rats [361]. Antibiotics targeting gut microbiota effectively prevented thermal and mechanical hyperalgesia caused by chronic constriction injury, reducing pro-inflammatory Th1 cells while increasing anti-inflammatory regulatory T cells. Removing regulatory T cells reversed this protective effect, suggesting that gut microbiota modulates inflammation and pain hypersensitivity [362]. Distinct differences in nociceptive responses between mouse strains were also observed. C57BL/6 mice developed hypersensitivity following spinal nerve ligation, associated with increased spinal dynorphin expression, while 129S6/SvEvTac mice failed to exhibit this response [363,364]. Additionally, paclitaxel-induced peripheral neuropathy appears linked to gut microbiota composition [365]. C57BL/6 mice developed mechanical, thermal, and cold hyperalgesia after paclitaxel treatment, whereas 129S6/SvEvTac mice remained resistant. Antibiotics protecting C57BL/6 mice from CIPN symptoms highlighted gut microbiota's role. Transplanting C57BL/6 microbiota into 129S6/SvEvTac mice conferred susceptibility to paclitaxel-induced hypersensitivity [365]. Paclitaxel increased *Alistipes onderdonkii* abundance while reducing *Akkermansia muciniphila*, a bacterium shown to attenuate neuroinflammation in Parkinson’s models and alleviate colitis-induced inflammation [366,367]. Similarly, oxaliplatin-induced mechanical allodynia was reversed by gut microbiota depletion using antibiotics. Sequencing revealed dysbiosis, with increased *Verrucomicrobia* and an altered *Bacteroidetes/Firmicutes* ratio, factors associated with chronic pain [368]. Elevated *Verrucomicrobia* abundance also correlated with chronic abdominal pain [369].

Antibiotics reduced macrophage infiltration into DRGs and lowered cytokine levels, including IL-6 and TNF-α, in oxaliplatin-treated mice. However, LPS from gut bacteria negated these neuroprotective effects, implicating TLR4 activation [370]. Promisingly, probiotics such as SLAB51 demonstrated efficacy in reducing hypersensitivity caused by paclitaxel, indicating a therapeutic potential for targeting microbiota to mitigate CIPN [371]. Further studies are essential to unravel the complex interactions between microbiota, neuroinflammation, and CIPN pathogenesis.

4.2.6 Neuroinflammation

A key pathophysiological mechanism associated with CIPN involves immune system activation, which leads to neuroinflammation and the degeneration of sensory nerve fibers. This process has been observed following treatments with paclitaxel or vincristine, where there is an infiltration of activated tissue-resident macrophages, particularly Langerhans cells, into the epidermis of the hind paw skin [372]. The presence of these cells in the skin is thought to contribute to the damage of intraepidermal nerve fibers due to the local production of neurotoxic cytokines [373]. Administration of the anti-inflammatory drug minocycline has shown protective effects against paclitaxel-induced degeneration of such fibers and mechanical hyperalgesia, highlighting its potential therapeutic benefits in CIPN treatment [374,375]. Oxaliplatin, another chemotherapeutic agent, has been found to activate cutaneous mast cells through the proteinase-activated receptor 2 (PAR2) pathway. This activation contributes to intraepidermal nerve fibers degeneration and the onset of mechanical allodynia. Prevention of oxaliplatin-induced peripheral neuropathy has been achieved by depleting mast cells or blocking PAR2 with the antagonist FSLLRY-NH_2, offering potential strategies to mitigate this form of neurotoxicity [376]. Beyond local inflammation, glial cells within the DRGs, including satellite glial cells, Schwann cells, and immune cells, also play a role in amplifying inflammatory responses and increasing neuronal excitability, which can lead to heightened pain sensitivity after chemotherapy [377]. In response to paclitaxel or oxaliplatin treatment, increased immunoreactivity to glial fibrillary acidic protein (GFAP), a marker for satellite and unmyelinated Schwann cells, has been observed in DRGs in animal models [378,379]. Additionally, a significant infiltration of macrophages has been noted in the DRGs, sciatic nerves, and dorsal horns of the spinal cord [380,381]. This macrophage infiltration is accompanied by an

upregulation of pro-inflammatory cytokines, including IL-1β and TNF-α, within both the DRGs and spinal cord dorsal horns [381–383]. Chemokines such as CCL2, CCL3, CCL4, and CCL11 are also overproduced in response to chemotherapy agents like paclitaxel and oxaliplatin, further promoting the inflammatory milieu within the DRGs and spinal cord [352]. Therapeutic interventions targeting this inflammatory cascade have proven effective in reducing the progression of CIPN. For instance, blocking the IL-1 receptor (IL-1ra) or administering anti-inflammatory cytokines like IL-10 via intrathecal injection, alongside the depletion of infiltrating macrophages through liposomal clodronate, resulted in a significant reduction of pro-inflammatory cytokine production. These strategies have demonstrated protective effects on the loss of IENFs and reversal of mechanical allodynia induced by paclitaxel [381,382]. In conclusion, both local immune activation and central glial responses play significant roles in the pathogenesis of CIPN. The modulation of these immune responses, through the use of anti-inflammatory agents or macrophage depletion, offers promising therapeutic avenues for alleviating the painful and debilitating symptoms associated with CIPN.

4.2.7 Inflammasome

Following nerve injury, inflammasome activation occurs within a few days [384]. Inflammasomes are multi-protein complexes made up of NOD-like receptors, pro-Caspase-1, and apoptosis-associated speck-like protein containing CARD. They are mainly categorized into groups such as NLRP1, NLRP3, NLRC4, IPAF, and AIM2 based on their components and serve as signaling platforms that mediate inflammatory responses and host defense mechanisms against microbial threats [377]. Initially identified as mediators of danger signals and pathogens, their role in pain perception, particularly inflammatory pain, has become more prominent in recent research (**Figure 7**). It is important to note that activation patterns of inflammasomes show distinct sex differences. In males, higher levels of NLRP1 and NLRP3 inflammasomes are observed, with NLRP3 playing a significant role in regulating pain perception in both sensory neurons and non-neuronal cells. In contrast, in females, inflammasomes like NLRP3 and AIM2 are more prevalent, and pain modulation is governed exclusively by sensory neurons, independent of non-neuronal cells [385,386]. Inflammasome activation facilitates the conversion of pro-Caspase-1 into its active form, Caspase-1, which then cleaves precursors of IL-1β and IL-18, generating mature inflammatory cytokines. These

events lead to cell death, neuronal excitability, and immune system overactivation, exacerbating central neuroinflammation by disrupting the blood-brain barrier. A significant portion of research on inflammasomes focuses on the upstream regulatory pathways of NLRP3. Thioredoxin-interacting protein (TXNIP), a key protein involved in oxidative stress and inflammation, interacts with the NLRP3 inflammasome to enhance its activation. The regulation of TXNIP in this context is tightly controlled through epigenetic mechanisms. For example, HDAC2 promotes deacetylation of histone H4, reducing miR-183 expression and counteracting its inhibition of TXNIP [387]. Additionally, TET2 has been shown to increase TXNIP expression at the transcriptional level, contributing to the progression of diabetic neuropathic pain [388]. Furthermore, the activation of TRP channels, including TRPV1, TRPA1, and TRPV4, has been shown to further stimulate NLRP3 inflammasome activity [389–391]. Pathological conditions, such as organelle damage or exposure to crystals, also facilitate the activation of the NLRP3 inflammasome. The inhibition of inflammasomes offers a potential therapeutic strategy for pain management. Markers related to inflammasome activity are commonly used to assess the effectiveness of such treatments [392]. However, it is crucial to acknowledge the potential side effects of therapies targeting inflammasome activity. Inflammasomes play an essential physiological role, particularly during the early stages of nerve injury, where Schwann cells and macrophages rapidly activate inflammasomes. This facilitates the clearance of axonal debris by macrophages and drives Schwann cells to promote nerve repair [393]. Moreover, IL-1β, a key inflammasome product, is critical in host defense against bacterial infection [394]. Thus, indiscriminate suppression of inflammasomes for pain relief could impair nerve regeneration and lead to immunosuppression, emphasizing the need for careful consideration of potential adverse effects when targeting inflammasome pathways for therapeutic purposes.

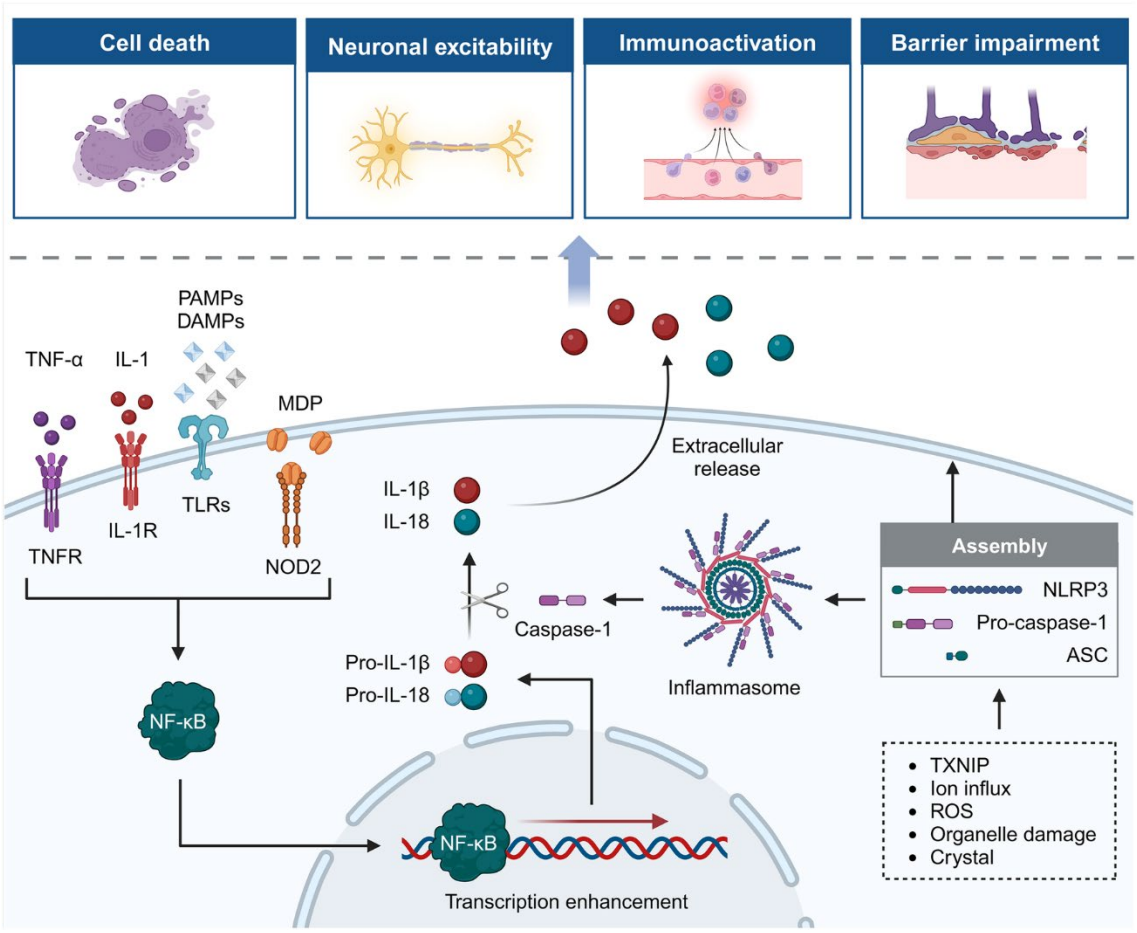


Figure 7: Overview on the inflammasome involvement in CIPN [15].

4.3 The role of the complement system in CIPN establishment and maintenance: a brief overview

Besides defending against infections, the complement system plays a vital role in linking innate and adaptive immunity by interacting with various immune cells, including dendritic cells, macrophages, and T and B lymphocytes [395]. Indeed, the complement system is crucial in immune defense, consisting several plasma proteins, which allow the clearance of pathogenic or damaged cells from the body, while promoting inflammation, tissue regeneration, and metabolic regulation, by enhancing the function of antibodies and phagocytes [396]. These complement proteins are produced primarily by the liver and circulate in an inactive form. Specific proteases activate them, initiating a cascade of immune responses that involve the release of cytokines [397,398]. Three pathways are responsible for activating the complement system, which eventually produces the same set of effector molecules: classical, alternative, and lectin [395]. In recent years,

attention has been paid to the role of complement, particularly the anaphylatoxin C5a, in pain regulation [399]. C5 itself is cleaved by C5 convertase, a serine protease, producing two fragments: C5a and C5b. While C5a plays an important role in pathogen clearance, its dysregulated activation can lead to various pathological states [400]. Indeed, C5a induces immune cells to produce pro-inflammatory cytokines and chemokines, while inhibiting anti-inflammatory cytokines [401]. C5a also increases the production of ROS by phagocytes, promotes calcium influx, and activates the NFkB pathway across various cell types, including neurons [402]. C5a is involved in the pathophysiology of peripheral neuropathy and neuroinflammatory pain, primarily through interactions with its two receptors expressed in immune and non-immune cells, C5aR1 and C5aR2, establishing the C5a/C5aR axis which promotes leukocyte's recruitment and release of cytokines and the production of a pro-inflammatory environment [403]. Emerging research highlights the critical role of the C5a/C5aR axis in neuroimmune interactions that contribute to the development and persistence of neuropathic pain hypersensitivity [404]. C5a contribute to this process by aiding the recruitment of neutrophils to the damaged tissue. The combination of nerve injury and immune cell infiltration induces functional changes in the nociceptive system, altering the expression of receptors, ion channels, and neurotransmitters, leading to the sensitization of nerve fibers at the injury site. Moreover, recent studies suggest that C5a/C5aR1 signaling is pivotal in the neuroinflammatory processes occurring in the damaged nerve, which trigger the onset of neuropathic pain, as the levels of C5a and C5aR1 were increased in the site of the injury [405,406]. The neuroimmune interaction mediated by complement activation and the subsequent release of C5a is involved in the development of neuropathic pain and hypersensitive anaphylactoid reactions, which are characterized by BBB leakage and plasma extravasation [407]. Thus, targeting the C5a/C5aR1 axis could be a promising strategy for pain management. Inhibition of C5a activity using C5aR antagonists has shown potential for controlling both acute and chronic neuropathic and neuroinflammatory pain [406]. Preclinical studies on taxane-induced CIPN have demonstrated that C5aR1 activation by paclitaxel is essential for the development and persistence of CIPN [406,408]. These findings suggest that C5aR1 inhibition could represent a novel therapeutic approach to managing CIPN and improving the quality of life for cancer patients undergoing therapy [409]. Interestingly, similar molecular mechanisms involving IL-8 expression and its receptors CXCR1/2 activation are common to both

taxane-induced and platinum compound-induced neuropathic pain, suggesting that these pathways may be shared across different chemotherapeutic agents [410].

CHAPTER V

5.1 Therapeutic strategies for pain management

The treatment of CIPN comprise a variety of approaches aimed at alleviating neuropathic pain, which often presents challenges due to its complex etiology and variable response to therapies [411]. Anti-inflammatory therapies provide an effective avenue for managing neuropathic pain, particularly with the use of non-steroidal anti-inflammatory drugs (NSAIDs) [412]. NSAIDs are often the first-line treatment, while opioids are reserved for cases where NSAIDs fail. Despite the theoretical benefits given by the link between inflammation and neuropathic pain, clinical trials focusing on NSAIDs for CIPN are sparse, necessitating more comprehensive investigations to clarify their role in this context. Regarding opioid therapy, as outlined by the German Society for Neurology, remains an option despite limitations such as tolerance development, side effects, and potential for misuse. Neuropathic pain typically responds moderately to opioids [274]. Among these, oxycodone has shown promise [413]. Its administration during chemotherapy was linked to a reduced incidence of CIPN. A phase IV multicenter trial further demonstrated that combining oxycodone and naloxone with anticonvulsants like gabapentin or pregabalin resulted in enhanced pain relief and better symptom control [414]. However, further studies are needed to confirm the long-term safety and efficacy of these combinations for CIPN management. Other approaches comprise, for instance, ion channel-targeted therapies and anticonvulsants. Ion channel dysfunction plays a pivotal role in the pathogenesis of CIPN, as chemotherapy-induced changes in peripheral nociceptors alter their excitability and ion channel activity [342]. Sodium, potassium, calcium, and TRP contribute significantly to the development of neuropathic pain. Experimental models have shown that lidocaine and mexiletine, which block these ion channels, can mitigate mechanical and cold allodynia caused by agents like oxaliplatin and vincristine [415,416]. Despite promising preclinical results, clinical trials, such as the phase I/II randomized double-blind study on lidocaine, revealed only moderate long-term analgesic effects, highlighting the need for further research to optimize these therapies [417]. Concerning anticonvulsants, these drugs, particularly gabapentin, have been explored for CIPN symptom management [15]. Pilot studies indicated potential benefits, with patients reporting improvements in self-perceived measures of neuropathy [418,419]. However, a phase III randomized, double-blind, placebo-controlled trial found no significant reduction in pain scores with gabapentin compared to placebo,

suggesting its limited efficacy in addressing primary outcomes like pain intensity or sensory dysfunction in CIPN [420]. Finally, neurotransmitter-based therapies, specifically targeting serotonin and norepinephrine reuptake, have emerged as effective strategies for neuropathic pain relief [15]. Serotonin-norepinephrine reuptake inhibitors (SNRIs) such as duloxetine suppress nociceptive signal transmission to the spinal dorsal horn [421]. Duloxetine is particularly effective in CIPN, as their effects have been evaluated assessing its use, for instance, in taxane-induced painful neuropathy [422]. A phase III randomized, double-blind, crossover trial demonstrated significant pain reduction in patients treated with duloxetine, especially those experiencing neuropathy due to platinum-based chemotherapy [423]. Comparative studies also revealed that duloxetine outperformed pregabalin in safety and efficacy for taxane-induced neuropathic pain. As a result, duloxetine is now a recommended therapy for CIPN. However, concerns about potential drug interactions, particularly those affecting hepatic metabolism, limit its widespread use and necessitate careful patient selection. Overall, while multiple therapeutic options are available, including opioids, anti-inflammatory agents, ion channel blockers, anticonvulsants, and neurotransmitter-based treatments, each approach has limitations. Ongoing research and clinical trials are crucial to refine these strategies, and address safety concerns to improve the quality of life for patients undergoing chemotherapy.

5.2 C5aR inhibitors

In recent years, the inhibition of C5aR has gained attention as a potential anti-inflammatory treatment. Despite extensive research, developing highly effective C5aR antagonists remains challenging due to unclear disease mechanisms and undesirable side effects [424]. An emerging alternative involves the use of allosteric modulators, which offer significant advantages, including enhanced safety profiles and drug-like properties [406]. These modulators selectively influence specific intracellular signaling pathways without interfering with the natural ligand's ability to bind to the receptor, unlike traditional peptidomimetic orthosteric inhibitors that replicate the C-terminal segment of the C5a [425,426]. Among cyclic peptidomimetics, PMX-53 (3D53) demonstrated nanomolar affinity for C5aR and effectively blocked immune and inflammatory pathways in preclinical models [427]. Administered via various routes, including intravenous and subcutaneous injections, PMX-53 showed promise for treating

inflammatory diseases such as psoriasis and rheumatoid arthritis. However, phase II trials revealed its limitations, including poor bioavailability and a short half-life [428]. Avacopan, formerly CCX-168, represents a significant advancement in this field. Approved in 2021, this oral C5aR antagonist is used to treat rare renal diseases like ANCA-associated vasculitis [429]. By serving as an adjunct to standard therapy, avacopan offers an effective alternative to high-dose glucocorticoids, minimizing the associated adverse effects due to its favorable safety and tolerability profile [430,431]. Avdoralimab (IPH5401), a monoclonal antibody targeting C5aR on neutrophils and myeloid-derived suppressor cells, reduces pro-inflammatory factor release and inhibits tumor growth [432]. Currently undergoing phase II trials, avdoralimab is being evaluated for its potential in treating bullous pemphigoid, a severe dermatological condition [433]. Despite promising results, no C5aR inhibitors have advanced in the treatment of neuropathies. Allosteric modulation of G-protein-coupled receptors (GPCRs), including C5aR, offers a groundbreaking approach for developing highly selective drugs. Extensive drug discovery programs to design allosteric modulators with improved pharmacological profiles compared to traditional orthosteric inhibitors have been made [434]. Using bioinformatics platforms, researchers identified DF2593A, a non-competitive allosteric inhibitor, which was characterized in various models, including those clarifying the C5a/C5aR axis's pathophysiological role [435]. These findings underscore the therapeutic potential of targeting the C5a/C5aR axis in CIPN.

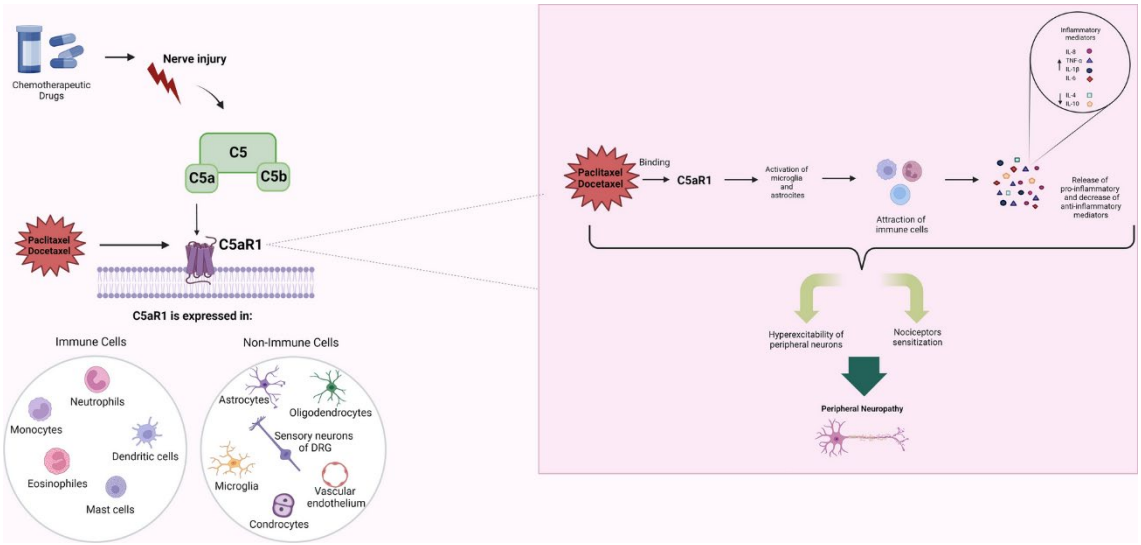


Figure 8: Schematic representation of the C5a/C5aR1 axis-related inflammatory process involved in CIPN and overview of the effects of the taxanes-C5aR1 binding [410].

5.3 Drug repurposing

The development of new drugs involves significant financial and time investments, with many candidates failing during clinical trials due to efficacy or safety concerns [436]. An alternative approach, drug repositioning, explores new therapeutic applications for already-approved medications reducing costs and time but also leveraging established safety profiles. Such a strategy is particularly promising for addressing adverse effects caused by other treatments, as occur in CIPN clinical research. Recent research has increasingly highlighted the potential of approved drugs in preventing CIPN. Studies investigating drug repositioning for CIPN generally fall into two categories. Mechanism-based studies rely on understanding the biological processes behind CIPN and matching them with the pharmacological actions of existing drugs. In contrast, screening-based studies use chemical libraries containing approved drugs to identify novel mechanisms for preventing or treating CIPN. This dual approach, combining mechanistic insights and broad screenings, provides a powerful framework for identifying effective therapies against CIPN. By repurposing drugs with known safety profiles, researchers aim to mitigate the high failure rates often seen in analgesic drug development while offering patients more efficient and safer treatment options [437].

CHAPTER VI

6.1 Aim

Paclitaxel, a chemotherapeutic agent renowned for its efficacy in controlling tumor proliferation, is often constrained in clinical applications due to severe adverse drug reactions [438]. Among these, peripheral neuropathy and hypersensitivity reactions (HSRs) represent significant challenges. HSRs, which can vary from mild discomfort to life-threatening anaphylaxis, frequently manifest within minutes of administration, particularly during initial or second exposures, impacting up to 40% of patients. While premedication with corticosteroids, antihistamines, and H2 antagonists can mitigate mild and moderate HSRs, these strategies are insufficient in preventing severe reactions, and desensitization protocols, although effective, fail to completely eliminate breakthrough events in some patients [439]. Regarding peripheral neuropathy, CIPN is a debilitating sensory disorder resulting from progressive and often irreversible peripheral nerve damage for which there is no definitive preventive or curative therapy despite extensive research efforts. Existing therapeutic options for CIPN are limited, focusing primarily on symptom management rather than prevention or reversal of the underlying pathology, failing to address the molecular and cellular mechanisms driving CIPN. Inflammation has been identified as a common denominator in paclitaxel-induced HSRs and CIPN [440]. This drug stimulates pro-inflammatory cytokines such as IL-8 and activates the complement system. IL-8, in particular, has emerged as a key mediator of paclitaxel-associated neuronal toxicity [441]. Investigating upstream mechanisms of IL-8 activation may provide new insights into the development of effective interventions for paclitaxel-induced side effects. Complement activation also results in the generation of the C5a, which exerts nociceptive effects via the G-protein coupled receptor C5aR1 [406]. Thus, the aim of this thesis is to investigate the mechanisms underlying paclitaxel-induced CIPN and anaphylaxis, with a particular focus on the C5aR1 and IL-8 pathways. By elucidating the molecular events driving these adverse reactions, this work seeks to identify potential therapeutic targets and contribute to the development of effective strategies to improve patient outcomes.

CHAPTER VII

7.1 Materials and Methods: *in silico* and *in vitro*

7.1.1 Bioinformatic analysis

Enrichment analysis was carried out using MetaCore™ (version 21.3, build 70600), a pathway and network analysis tool provided as part of the Cortellis platform by Clarivate. This software integrates a curated knowledgebase designed for analyzing high-throughput transcriptomic datasets. The resource encompasses 1,456 curated pathways describing protein interactions across human, mouse, and rat systems, covering signaling processes, metabolic networks, disease pathways, and stem cell mechanisms, all derived from peer-reviewed publications. The IL-8 protein from humans was used to query the database, with the analysis focused on identifying interactions under the parameters: Direction – Incoming; Effect – Activation; Mechanism – Expression influence and Transcriptional regulation. Additionally, the GOSTAR database (GVK Biosciences Private Limited, Plot No. 28A, IDA Nacharam, Hyderabad, India, 2010, https://gostardb.com/gostar/), developed by Excelra Knowledge Solutions, was consulted. GOSTAR is an online scientific repository that provides information on both published and patented inhibitors for various biological targets, along with corresponding structure-activity relationship (SAR) data. This database was employed to identify established targets of paclitaxel for subsequent analysis.

7.1.2 Functional assay

A cell-based cAMP assay, supplied by DiscoverX (catalog codes 86-0007P-2277AG for C5aR1 and 86-0007P-2818AG for C5aR2), was utilized for the study. This assay employs a stable cell line expressing non-tagged GPCRs to monitor receptor activity. GPCR activation through Gi- and Gs-coupled secondary messenger pathways was evaluated using the Hit Hunter® cAMP assay. This homogenous, non-imaging assay leverages DiscoverX's Enzyme Fragment Complementation (EFC) technology, which utilizes β-galactosidase (β-Gal) as the reporter molecule for functional readouts. The IC50 values were determined by fitting concentration-response data to a non-linear regression model.

7.1.3 SPR binding assay

Surface Plasmon Resonance (SPR) experiments were conducted using a BIAcore X100 instrument (Cytiva). To investigate interactions between paclitaxel or docetaxel and the receptors C5aR1 or C5aR2, anti-c-Myc and anti-Histidine antibodies were immobilized on a CM5 sensor chip via standard amine-coupling chemistry. Recombinant human C5aR1 tagged with c-Myc or C5aR2 tagged with 6xHistidine (10 µg/ml in 50 mM HEPES, pH 7.0, supplemented with 0.01% cholesteryl hemisuccinate Tris salt, 0.1% CHAPS, and 0.33 mM synthetic phospholipid blend DOPC:DOPS (7:3 w/w), Avanti Polar Lipids) were injected over the respective antibody surfaces, resulting in immobilizations of 849 RU and 1190 RU. Surfaces with only anti-c-Myc or anti-Histidine antibodies served as controls and for blank subtraction. For studies examining interactions between paclitaxel or docetaxel and C5a or C5a des-Arg, human recombinant proteins (20 µg/ml) were immobilized on a CM5 chip using the same amine-coupling protocol, achieving immobilizations of 1913 RU and 1520 RU, respectively. To analyze the binding of paclitaxel or docetaxel to anti-C5aR1 antibody (CD88 clone S5/1), the monoclonal antibody (20 µg/ml) was immobilized on a CM5 chip, yielding 4015 RU. Binding assessments were conducted in single-cycle mode. Paclitaxel and docetaxel were prepared at 200 µM in running buffer (for C5aR1 and C5aR2) or in 10 mM HEPES (pH 7.4) containing 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (HBS-EP+) for experiments involving C5a and C5a des-Arg. The compounds were injected for 2 minutes over various surfaces, followed by dissociation monitoring. To determine kinetic parameters for paclitaxel or docetaxel on C5aR1, the compounds were injected at increasing concentrations over C5aR1 and control surfaces, with sensorgrams analyzed using BIAevaluation software (version 3.2) based on a single-site nonlinear fitting model. Only sensorgrams with χ^2 values near 10 were included. Additionally, equilibrium dissociation constants (Kd) were calculated by applying Scatchard's equation to the plot of bound RU versus ligand concentration. For competition assays, 50 nM C5aR1 was mixed with increasing paclitaxel or docetaxel concentrations and injected over C5a, C5a des-Arg, and CD88 surfaces. Reagents included recombinant C5aR2 (CUSABIO), recombinant C5aR1 (Origene), anti-6X His antibody (Abcam), anti-c-Myc antibody (Santa Cruz Biotechnology), CD88 antibody (Biolegend), and complement C5a des-Arg (Merck).

7.1.4 Cell cultures

Cell cultures were routinely tested for Mycoplasma contamination using the Mycoplasma PCR Detection Kit (Applied Biological Materials, USA). The F11 hybridoma cell line (ECACC 08062601), a model for DRG neurons, was maintained in DMEM (Euroclone, MI, Italy) supplemented with 10% FBS (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Euroclone), and 1% glutamine (Euroclone) at 37°C in a humidified 95% air-5% CO2 atmosphere. Cells were seeded at 1 × 10^4 cells/cm² and used at passage 18. After 24 hours, differentiation was induced by adding rat NGF (rNGF) (Sigma) at a concentration of 50 ng/ml, dissolved in DMEM containing 1% penicillin/streptomycin and 1% glutamine (without FBS). Medium was refreshed every 3 days until complete differentiation, which occurred after 7 days.

DRG neurons were isolated by dissociating ganglia from postnatal (P2) Sprague Dawley rats (Envigo, Bresso, Italy) using established protocols [49, 50]. To reduce contamination, long roots were removed from DRGs. The ganglia underwent enzymatic dissociation with 1.5 mg/ml papain for 20 minutes at 37°C, followed by 4 mg/ml collagenase for 30 minutes at 37°C. Cells were then mechanically dissociated with a fire-polished glass Pasteur pipette to form a single-cell suspension. The dissociated cells were plated on 96-well plates coated with Laminin (10 µg/mL overnight at Room Temperature). After 1 week in DMEM with high glucose, supplemented with 10% FBS, 1× N2, 1× B27, 100 IU/ml penicillin, and 10 mg/ml streptomycin, the cells were treated based on the experimental design.

Mouse RAW 264.7 macrophages (ATCC TIB-71) were cultured according to the manufacturer's protocol in DMEM supplemented with 10% FBS (non-heat inactivated) (ATCC, USA) and seeded at 1 × 10^4 cells/cm². After 24 hours, cells were exposed to C5a (10 nM, R&D, USA) or PAC (10 nM, Sigma, USA) in the medium for another 24 hours. The media was replaced with fresh culture media (5 ml) for 24 hours and collected for proteome profiling.

Human THP-1 monocytes (ATCC TIB-202) were cultured in RPMI with 10% FBS (non-heat inactivated), 1% glutamine, and 2-mercaptoethanol (0.05 mM). Differentiation was induced by 20 ng/ml PMA for 72 hours. Following differentiation, cells were treated as described, with the media replaced and collected for proteome analysis.

7.1.5 Drug treatments

Stock solutions of paclitaxel (Sigma-Aldrich; 10 mM) and C5a (R&D Systems, Inc. MN, USA; 20 mM) were created by dissolving the powders in DMSO, with aliquots stored at −20 °C and −80 °C, respectively. The IgG2B antibody, C5aR antibody (BioLegend, CA, USA), C5aR1 antagonist (MBS691011, MyBioSource, USA), and Avacopan (Selleckchem, USA) were provided in ready-to-use formulations. DF3966A (Dompé farmaceutici S.p.A., L'Aquila, Italy) was prepared as a 20 mM stock solution in DMSO 100%.

7.1.6 Western blotting

F11 cells underwent 24-hour treatments with either paclitaxel (10 nM), C5a (10 nM), IgG2B antibody, or C5aR antibody (50 nM), individually or in combination with paclitaxel or DF3966A (10 μM). For time-course experiments, differentiated F11 cells were exposed to paclitaxel (10 nM) or C5a (10 nM) for shorter durations of 15, 30, and 90 minutes. After treatment, cells were collected and lysed using ice-cold RIPA buffer, prepared with phosphate-buffered saline (pH 7.4), supplemented with 0.5% sodium deoxycholate, 1% Igepal P-40, 0.1% SDS, 5 mM EDTA, and protease/phosphatase inhibitor cocktails (Sigma-Aldrich, USA). Protein extracts (30 μg) were resolved using Bolt precast 4–12% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo, USA). To block nonspecific binding, membranes were incubated with 5% Blotto dry milk (Santa Cruz, USA) dissolved in 1× Tris-buffered saline with Tween 20 (TBS-T) for 1 hour at room temperature. For C5aR1 detection, membranes were incubated overnight at 4°C with rat anti-C5aR1 antibody (1:200; sc-53788, Santa Cruz Biotechnology) diluted in TBS-T containing 5% milk. Peroxidase-conjugated anti-rat IgG (1:10,000; Sigma) was employed as the secondary antibody. For the other detected proteins, membranes were incubated overnight at 4 °C with the following primary antibodies, diluted in the blocking solution: rabbit anti p-Fak 1:1000 (Tyr397, 3283S, Cell Signaling, USA), rabbit anti p-STAT3 1:1000 (Tyr705, 9131S, Cell Signaling, USA), rabbit anti p-cortactin 1:500 (Abcam, UK), rabbit anti cortactin 1:500 (ab81208), rabbit anti-JAK2 (3230S, Cell Signaling), anti-p-Jak2 1:200 (Tyr 1007/Tyr 1008, sc16566, Santa Cruz, USA), rabbit anti p-NFkB 1:1000 (Ser536, ab239882, Abcam), rabbit anti NFkB 1:1000 (ab16502, Abcam), rabbit anti p-c-Fos (5348T, Ser325348, Cell Signaling), rabbit c-Fos 1:1000 (2250T, Cell Signaling), rabbit

anti-p-Jun 1:1000 (T93, ab81319, Abcam), anti-c-Jun 1:1000 (ab32137, Abcam), rabbit anti p-P38 1:1000 (Thr180/Tyr182, 9211S, Cell Signaling), rabbit anti-p38 1:1000 (9212, Cell Signaling), rabbit anti-p-MAPK6/ERK3 1:1000 (S189, ab74032, Abcam), rabbit anti-MAPK6/ERK3 1:1000 (ab53277, Abcam). Secondary detection utilized peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:30,000; Invitrogen, USA). Membranes were stripped for reprobing using Restore Western Blot Stripping Buffer (Thermo Scientific) as per the manufacturer's protocol. Signals were detected using ECL reagents (Thermo, USA) and visualized with a Uvitec imaging system (UK). Digital images were analyzed, and relative band densities were quantified and normalized to actin using ImageJ software. Results were expressed as relative units (RU).

7.1.7 Quantitative real-time PCR

For the analysis of gene expression, total RNA was isolated using Trizol reagent following the manufacturer's guidelines. RNA concentration was measured spectrophotometrically in RNAase-free water. 1 µg of total RNA was converted into cDNA with the ProtoScript First Strand cDNA Synthesis Kit (NEB). RT-PCR was conducted using the ABI 7300HT sequence detection system (ABI) in a final volume of 20 µl, including EagleTaq Universal Master Mix (Roque), DEPC water, 4 µl of cDNA, and the following qPCR assays: *CXCL1* Mm04207460m1 (Applied Biosystems, USA), *TRPV4* ID qRnoCIP0027857, and *TRPV1* ID qRnoCIP0024978 from Biorad (Biorad Laboratories, USA). Each gene was tested in triplicate. The internal control gene *GAPDH* (ID qRnoCIP0050838, Biorad, USA) was used to normalize gene expression. The RT-PCR program included a 3-minute pre-heating at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and a final 10 s at 65°C. Relative gene expression was calculated using the ΔΔCt method to determine fold expression differences, as previously described.

7.1.8 Electrophysiological recording

For 7 days, DRG-derived neurons were cultured with different treatments: paclitaxel (10 nM), paclitaxel combined with DF3966A (1 µM final concentration), C5a (100 nM), and C5a combined with DF3966A. The compounds were applied for either 1 min 30 s (acute stimulation) or 5 min (chronic stimulation). After application, a wash-out with a

physiological control solution was performed for an equivalent duration (around 10 min). Electrophysiological recordings were conducted using the patch-clamp technique in whole-cell mode. The extracellular solution contained NaCl 135 mM, KCl 2 mM, CaCl2 2 mM, MgCl2 2 mM, HEPES 10 mM, glucose 5 mM, with pH adjusted to 7.4. The pipette solution included potassium aspartate 130 mM, NaCl 10 mM, MgCl2 2 mM, CaCl2 1.3 mM, EGTA 10 mM, and HEPES 10 mM, pH 7.3. Data were collected using pClamp 8.2 software and the MultiClamp 700A amplifier (Axon Instruments, CA, USA) in current-clamp mode.

7.1.9 Proteome profiler assay

M0 (differentiated, not stimulated) RAW 264.7 and THP-1 cells were exposed to C5a (10 nM, diluted in culture medium), paclitaxel (10 nM, diluted in culture medium), DF3966A (1 μM), Avacopan (1 μM), or C5aR1 antagonist (1 μM) for 24 hours. After incubation, the cells were gently washed with sterile PBS, and the culture media was replaced with fresh media (5 mL) for an additional 24 hours before collection. For control conditions, cells were kept in culture media for 24 hours, followed by a 24-hour incubation with fresh media before collection. The conditioned media were then centrifuged to remove particulate matter and immediately analyzed.

Sample volumes were adjusted according to the manufacturer's recommendations (500 μL). The reagents were prepared following protocols from R&D Systems (USA). Membranes were first incubated with Array Buffer 6 for 1 hour on a rocking platform. Samples were prepared by adding 1 mL of Array Buffer 4 to separate tubes and adding 15 μL of reconstituted mouse cytokine detection antibody cocktail (>40 mouse cytokines) to each sample, followed by a 1-hour incubation. After removing the Array Buffer 6, the antibody-sample mixtures were applied to the membranes and incubated overnight at 4°C. The membranes were washed three times the next day, followed by incubation with Streptavidin-HRP (1:2000) for 30 minutes at room temperature. After washing the membranes again, 1 mL of Chemi Reagent mix was added to each membrane. Multiple exposures were captured using a UVITEC digital analyzer (Alliance, Cambridge, UK). The positive signals in the developed membranes were identified by aligning the transparency overlay template with the reference spots. The pixel densities of each cytokine pair were analyzed using ImageJ, and the average background was subtracted. For human macrophages, the human XL Cytokine Array kit

(#Ary022B) was used, and for murine macrophages, the mouse Cytokine Array Panel A (#Ary006) was used (R&D Systems, USA).

7.2 Matherials and methods: *in vivo*

7.2.1 Animals

Animal care and experimental procedures complied with both national and international standards (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines [52], and the Basel declaration, including the 3R principles). Behavioral tests were conducted exclusively between 9:00 am and 1:00 pm, with each mouse participating in a single test. Efforts were taken to minimize animal distress, and cervical dislocation was performed at the conclusion of all procedures. The behavioral studies were performed on male Balb/C mice (6–8 weeks, Charles River) housed in the animal care facility of the Department of Pharmacy, University of Naples.

C5ar1−/− mice (Jax mice stock number 006845) and BALB/cJ control males (PubMed: 8779720) were also used. The C5ar1−/− colony was maintained at the Ribeirao Preto Medical School, University of Sao Paulo, in adherence to ethical standards for animal research. Animals were housed under stable conditions, with controlled temperature (22 ± 1 °C), humidity (60 ± 10%), and a 12-hour light/dark cycle, with ad libitum access to food and water.

Experiments were designed to use the smallest number of animals necessary to achieve reliable results, avoiding redundant tests, and aligning with the 3Rs (Replace, Reduce, Refine). Sample sizes were calculated using G*Power to ensure adequate statistical power for detecting significant effects. Randomized allocation of mice to treatment groups was implemented, and the investigator conducting the experiments and assessing outcomes was blinded to the group assignments.

All animals were included in the final analysis, with each experiment conducted once to ensure ethical and efficient use of resources while maintaining scientific rigor.

7.2.2 Drug treatments

For the CIPN model, paclitaxel solution (Onatax®, 100 mg/16.7 ml; cod 018155) was generously provided by Prof. Thiago M. Cunha (Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Brazil). DF3966A (Dompé farmaceutici S.p.A., L'Aquila, Italy) was dissolved in water (30 mg/10 ml) and administered orally at 30 mg/kg every 12 hours for 14 days during CIPN induction. On days 1, 3, 5, and 7, DF3966A was given one hour post-paclitaxel injection. For the HSR model, paclitaxel (Selleckchem, USA, cod. S1150) was intravenously injected at 20 mg/kg (0.1 mL/kg), prepared in Cremophor EL (polyoxyl 35 castor oil, Sigma-Aldrich, cod. 238470) or PEG400/TWEEN80 (Sigma-Aldrich). DF3966A (30 mg/kg, os) or its saline vehicle was administered three hours prior to paclitaxel. Avacopan (30 mg/kg, os) or its vehicle (PEG-400/solutol-HS15 70:30, 5 mL/kg [29]) was administered one hour beforehand.

7.2.3 Induction of peripheral neuropathy by paclitaxel

The method, adapted from Polomano et al., 2001, was modified for mice. A paclitaxel solution (100 mg/16.7 ml) was diluted in saline to achieve a 4 mg/kg dose [442]. Animals received intraperitoneal injections (0.3 ml) on alternate days (1, 3, 5, and 7). Measurements were taken four hours after each paclitaxel administration.

7.2.4 Mechanical allodynia

Mechanical allodynia and sensitization changes were evaluated using the Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Italy). Mice were placed in a chamber with a mesh metal floor under a plastic dome, allowing free movement while preventing jumping. After acclimation in a quiet room for 15–30 minutes, a mechanical stimulus was applied to the mid-plantar region of the hind paw, with the force at which withdrawal occurred recorded automatically. A 5 g cut-off was established. Testing was conducted three hours post-treatment with DF3966A on days 1, 3, 5, 7, 10, and 14.

For *C5ar1*$^{-/-}$ mice and wild-type controls, von Frey filaments were used to measure mechanical allodynia. A graded series of filaments was applied to the right hind paw, with the lowest force eliciting flinching recorded as the mechanical threshold. Results

are presented as the logarithm of the threshold. Measurements were taken four hours after paclitaxel administration during the treatment period.

7.2.5 Cold allodynia

Cold sensitivity was assessed by counting paw withdrawal reactions following acetone application to the dorsal paw surface [443]. Using a syringe connected to a polyethylene tube, 50 µL of acetone was gently applied to the paw. A response such as paw lifting, licking, or flinching upon contact with acetone indicated cold allodynia. Measurements were taken three hours post-treatment with DF3966A on days 1, 3, 5, 7, 10, and 14.

In both *C5ar1−/−* mice and their wild-type counterparts, withdrawal responses were recorded using a chronometer over a 2-minute observation period after acetone application. Behavioral testing for cold sensitivity was performed approximately 15 minutes after the mechanical stimulation test.

7.2.6 Real-time PCR analysis on paws and spinal cord

On day 15, paw and spinal cord samples were collected for RNA extraction using TRIzol Reagent (Bio-Rad Laboratories), following the manufacturer's protocol. From 2 µg of total RNA, cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). Quantitative PCR was carried out on a Bio-Rad CFX96 Connect Real-Time PCR System, with conditions set at 95°C for 15 minutes, followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C. Each reaction contained 20 ng of cDNA, 2X QuantiTect SYBR Green PCR Master Mix, and specific primer pairs to amplify *c5aR1* (Cat.n° QT00288232), *TRPV1* (Qiagen ID QT00167048), *TNF-α* (ID QT00104006), *Il1β* (ID QT01048355), and *IL6* (ID QT00098875) in a 50 µl final volume. β-Actin was used as the reference gene, and relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

7.2.7 Assessment of vascular leakage

To evaluate vascular leakage and skin manifestations associated with paclitaxel-induced HSRs, Evans Blue (EB, 0.8%/0.3 mL/iv) was administered to mice 30 minutes after

paclitaxel injection. The blue coloration of the ears was scored from 0 to 5, where "0" indicated no visible blue area and "1 to 5" represented the extent of visible blue, as previously described.

7.2.8 Plasma extravasation

Approximately 40 minutes post-paclitaxel administration, the animal was euthanized, and its ears were excised, minced, and placed in 2 mL formamide. After overnight incubation at 55°C, centrifugation was performed, and the supernatant was analyzed spectrophotometrically at 620 nm. Formamide served as a blank in duplicate, and results were normalized to tissue weight (µg/mg).

7.2.9 ELISA assay

Blood was collected via intracardiac injection into tubes and left at room temperature. After 30 minutes, serum was isolated by centrifugation at 2000 × g for 10 minutes at 4°C. Aliquots were stored at −20°C, and histamine and SC5b-9 levels were analyzed using an ELISA kit (Mybiosource, San Diego, USA).

7.3 Data analysis

The data are expressed as mean ± SEM and analyzed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Behavioral experiments were evaluated using one- or two-way ANOVA, followed by Bonferroni or Tukey post hoc tests for multiple comparisons, as appropriate. Sham vehicle and sham DF3966A mice showed no significant differences; thus, a single sham group is reported. Statistical significance was set at $*P < 0.05$. For hypersensitivity reaction (HSR) experiments, one-way ANOVA followed by Dunnett's test was applied for group comparisons. *In vitro* data were analyzed using one-way ANOVA with Tukey's post hoc test. All animals and samples were included in the analysis, with no exclusions.

CHAPTER VIII

8.1 Results

8.2 Screening of the identified receptors and prediction of C5aR1-paclitaxel binding

An *in silico* approach was applied to select optimal paclitaxel targets, based on bioinformatic analysis and literature review. To obtain 3D receptor structures (**Table 1**), the Comprehensive Therapeutic Target Database (CTTD), the Protein Data Bank (PDB), and AlphaFold models for retrieval were utilized.

Target	*Predicted affinity*
TLR2_HUMAN	High
NK1R_HUMAN	High
EDNRB_HUMAN	High
EGFR_HUMAN	High
C5AR1_HUMAN	**High**
EDNRA_HUMAN	High
PAR2_HUMAN	Low
C5AR2_HUMAN	Low
AA2BR_HUMAN	Low
RET_HUMAN	Low
CRFR2_HUMAN	Low
TNR1A_HUMAN	Low
S1PR1_HUMAN	Non-bound protein
PAR1_HUMAN	Non-bound protein
KIT_HUMAN	Non-bound protein
ADRB2_HUMAN	Non-bound protein
TLR8_HUMAN	Non-bound protein
LPAR1_HUMAN	Non-bound protein
TGFR2_HUMAN	Non-bound protein
DDR1_HUMAN	Non-bound protein
NTR1_HUMAN	Non-bound protein
PTAFR_HUMAN	Non-bound protein
C3AR_HUMAN	Non-bound protein
PE2R2_HUMAN	Non-bound protein
ACM3_HUMAN	Non-bound protein
PE2R3_HUMAN	Non-bound protein
PE2R4_HUMAN	Non-bound protein

Table 1 Selected potential paclitaxel targets and their predicted affinity.

The 3D protein structures were generated using the Protein Preparation Wizard from Schrodinger, and docking of paclitaxel was conducted using LiGen™ in the identified binding sites [444]. For GPCRs, when only apo structures were available, binding sites were determined by alignment with holo forms of other GPCRs. Molecular docking simulations performed via Exscalate revealed that six proteins exhibited a high affinity for paclitaxel, with a significant preference for the GPCR class (**Table 1**).

C5aR1 was identified as a high-affinity target among the proteins analyzed. Using previously acquired data and X-ray structural analysis, the interaction between C5aR1 and paclitaxel was examined, revealing three primary binding sites: one orthosteric and two allosteric [445]. The 6C1R pdb model was selected for molecular docking due to its higher resolution, ensuring more reliable computational results. This model underwent structural and chemical refinement for increased accuracy. Docking paclitaxel into the orthosteric binding site of the 6C1R structure revealed a notable binding pose, with multiple interactions observed with residues such as Arg175, Tyr192, Lys279, and others (**Figures 9A, B**). These findings were unexpected, as C5aR1 had not been previously recognized as a paclitaxel target, as confirmed by the target list from the GoStar database (GVK BIO Online Structure Activity Relationship Database) (data not shown).

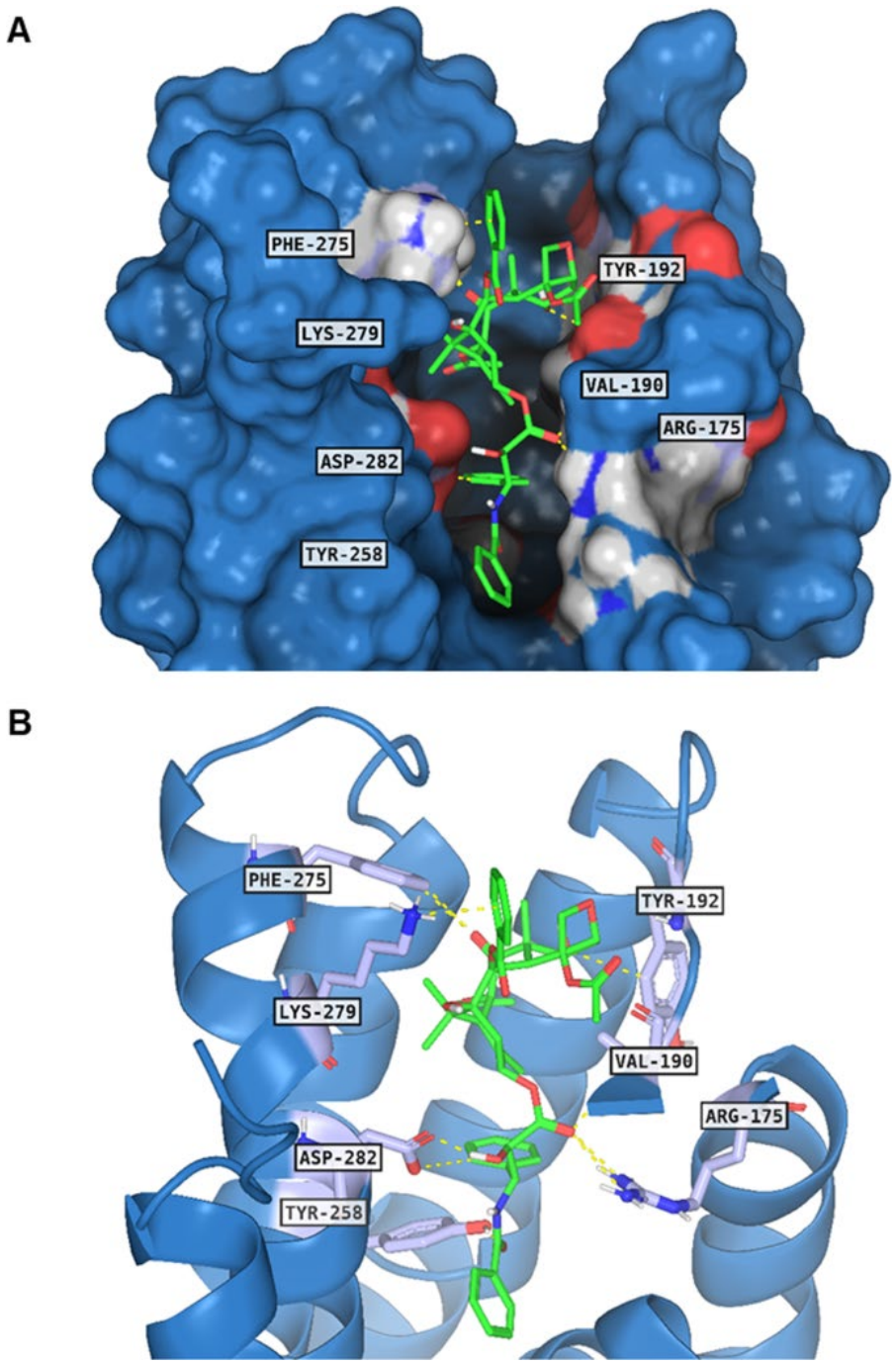


Figure 9: Paclitaxel binding mode in C5aR1 binding site. *The ligand between C5aR1 and paclitaxel is reported in green lines, while the protein structure is reported in blue surface (A) and cartoon (B). The contact residues and related hydrogen bonds are depicted in slate sticks and yellow dots, respectively.*

Considering the C5a-C5aR1 axis's involvement in immune responses and CIPN, C5aR1 was chosen for further exploration to assess its interaction with paclitaxel and potential contribution to paclitaxel-induced CIPN.

8.3 *In vitro* studies confirmed paclitaxel-C5aR1 binding

To confirm the *in silico* predictions, Surface Plasmon Resonance (SPR) analysis was carried out. The recombinant human C5aR1 was immobilized on a CM5 sensorchip, while paclitaxel was allowed to flow across it. Binding of paclitaxel to C5aR1 was observed, showing dose-dependent interaction. The dissociation constant (Kd) was found to be 670 nM (**Figure 10B**), and the equilibrium affinity value, measured by steady-state binding, was 525 nM (data not shown). *In silico* data, comparing paclitaxel's binding mode to both C5aR1 and C5aR2, revealed that paclitaxel cannot bind C5aR2 in the same manner as it does C5aR1 (data not shown). Additionally, paclitaxel failed to bind immobilized C5aR2, C5a, or C5a des-Arg proteins, confirming that the interaction was specific to C5aR1 (**Figure 10A**). These results validate the selective affinity of paclitaxel for C5aR1.

To further explore the interaction between paclitaxel and C5aR1, co-injections of C5aR1 (50 nM) with increasing concentrations of paclitaxel were performed on surfaces of C5a or C5a des-Arg. Paclitaxel was observed to inhibit C5aR1 binding to both C5a and C5a des-Arg in a dose-dependent manner, with IC50 values of 4.579 μM (**Figures 10C, D**) and 2.134 μM (**Figures 10E, F**), respectively. To verify the specific binding of paclitaxel to C5aR1's binding site for its ligand, competition experiments were conducted using a monoclonal anti-C5aR1 antibody known to neutralize C5aR1 and C5a interaction (data not shown). Paclitaxel was found to inhibit this interaction in a dose-dependent manner, yielding an IC50 of 3.632 μM (**Figures 10G, H**). To assess whether paclitaxel activates or inhibits C5aR1, cell-based cAMP assays were performed, revealing that paclitaxel activates C5aR1 but does not inhibit it. Dose-response experiments demonstrated that paclitaxel's EC50 is 5.81 μM with an Emax of approximately 50%, confirming paclitaxel as a partial agonist of C5aR1 (data not shown).

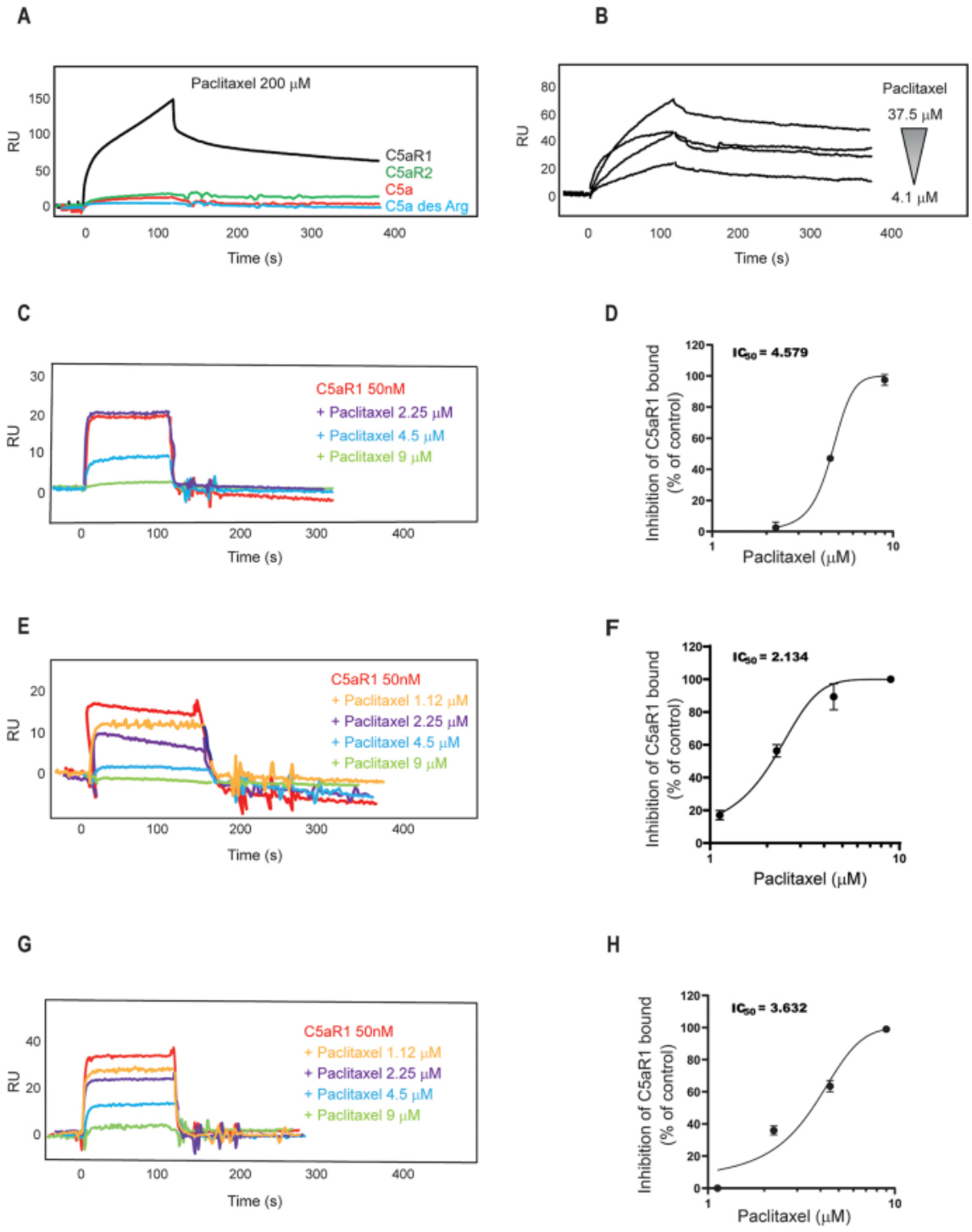


Figure 10: Paclitaxel and C5aR1 binding studies by SPR. *A) Binding of paclitaxel on immobilized C5aR1, C5aR2, C5a, and C5a des-Arg; B) Dose-dependent binding of paclitaxel on immobilized C5aR1; C, D) Co-injection of paclitaxel and C5aR1 on immobilized C5a and relative IC50; E, F) Co-injection of paclitaxel and C5aR1 on immobilized C5a des-Arg and relative IC50; G, H) Co-injection of paclitaxel and C5aR1 on immobilized monoclonal antibody anti-C5aR1 and relative IC50.*

The results indicate that paclitaxel specifically binds to and activates C5aR1 in a competitive manner, suggesting its involvement in the side effects associated with paclitaxel. Additionally, SPR assays were performed to examine whether docetaxel, another chemotherapeutic agent from the taxane class, could influence the interaction between C5aR1, C5aR2, and their natural ligands (C5a and C5a des-Arg). These experiments (data not shown) showed that, similarly to paclitaxel, docetaxel also binds to C5aR1 in a dose-dependent manner.

8.4 C5aR1 is a key mediator of paclitaxel-induced CIPN *in vitro*

Following the identification of C5aR1 as a novel paclitaxel target, the potential intracellular pathways activated upon binding and C5aR1's role in paclitaxel-induced neurotoxicity were explored. Thus, the expression of NFkB, c-Jun and c-Fos, p38 and ERK3/MAPK6 was investigated in differentiated F11 cells following paclitaxel treatment (**Figure 11)**. *In vitro* studies using F11 cells revealed that paclitaxel's interaction with C5aR1 activates the NFkB/p38 signaling pathway and c-Fos expression with induced activation of NFkB (**Figures 11A and F**), significantly increased expression of the phosphorylated forms of Jun and c-Fos (**Figures 11B, C and F**), which are the two canonical AP-1 transcription factors, and of the p38 (**Figures 11D and F**), while it did not affect the expression of another MAPK family member, p-ERK3/MAPK6 (**Figures 11D and F**). Interestingly, the co-administration of a C5aR1 inhibitor, DF3966A at 10μM for 24 hours, completely counteracted paclitaxel-induced upregulation of p-NFkB, p-c-Fos and p-p38 (**Figures 11A, C, D and F**), while it did not affect p-Jun and ERK3/MAPK6 expression (**Figures 11B, E and F**). These data suggest that C5aR1 is crucial for the paclitaxel-induced activation of NFkB/p38 pathway and c-Fos, which are known to be involved in the downstream activation of the IL-8 signaling.

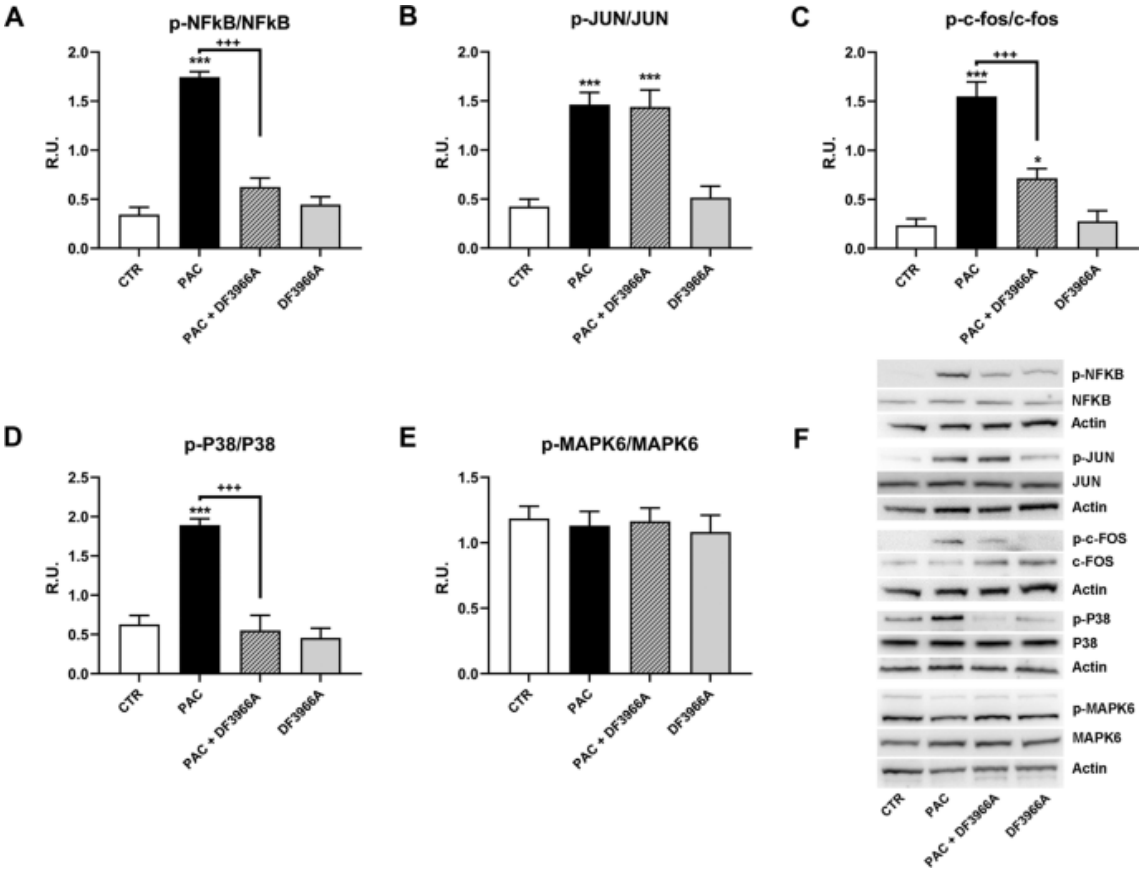


Figure 11: Paclitaxel-C5aR1 intracellular pathway. *Quantification of the expression of phosphorylated and total forms of NFkB(A), c-Jun (B), c-Fos (C), p38 MAPK (D) and ERK3/MAPK6 (E) in differentiated F11 cells following paclitaxel (PAC) treatment evaluated by western blot analysis (F). F11 cells were treated for 24 h with PAC (10 nM) and DF3966A (10 μM). ***P < 0.0005; *P < 0.05 vs CTR; +++P < 0.0005 vs paclitaxel. Data are mean ± SEM of n = 3 different experiments.*

The inhibition of C5aR1 provided significant protection against paclitaxel-induced neuropathological effects *in vitro* (**Figures 12, 13**). Given that CXCL1 (i.e., murine IL-8) mediates paclitaxel-induced neuronal toxicity in F11 sensory neurons and that the activation of C5aR1 by paclitaxel is upstream of IL-8/CXCL1 pathway, whether and how the activation and the inhibition of C5aR1 would affect the expression of key pain markers in F11 cells was investigated. With this aim, the protein expression of C5aR1 (**Figure 12A**) in differentiated F11 cell line was assessed, and then these cells were treated for 24 hours with paclitaxel (10nM) or C5a (10nM), the natural ligand of C5aR1. Western blot experiments revealed that both paclitaxel and C5a elevated p-cortactin expression, a protein critical for modulating synaptic plasticity and terminal axonal

arborization. Similarly, p-FAK levels, important for microtubule stabilization, were increased under the same conditions (**Figures 13A and B**), indicating that neuronal plasticity is influenced by these stimuli. Furthermore, both treatments enhanced p-JAK2 and p-STAT3 protein levels compared to control conditions (**Figures 13C and D**), illustrating their shared ability to activate the JAK-STAT pathway, a mechanism linked to neuropathic pain and neuronal remodeling. In F11 cells, paclitaxel and C5a stimulation significantly upregulated CXCL1 mRNA after 20 hours (**Figure 12B**). At 24 hours, paclitaxel also elevated CXCL1 protein levels, suggesting a sequential activation of the CXCL1-mediated pathway initiated by C5aR1 signaling. To confirm whether these effects were dependent on C5aR1 activation, experiments were conducted using an anti-C5aR1 antibody (C5aR1 Ab, 50nM) in combination with paclitaxel. The antibody effectively counteracted the paclitaxel-induced increase in p-FAK and p-JAK2 protein levels (**Figure 14**), emphasizing C5aR1's central role in mediating neurotoxic effects. Additionally, treatment with DF3966A, a C5aR1 antagonist, reversed paclitaxel-induced increases in p-FAK and p-cortactin levels, restoring them to control values (**Figure 13E**). The same antagonist also reduced p-JAK2 and p-STAT3 protein levels (**Figure 13F**) and lowered TRPV1 and TRPV4 expression, which are crucial mediators of thermal, chemical, and mechanical stimuli in nociception, as shown by real-time PCR (**Figure 13G**). These findings underscore the therapeutic potential of targeting C5aR1 to mitigate paclitaxel-induced neurotoxicity.

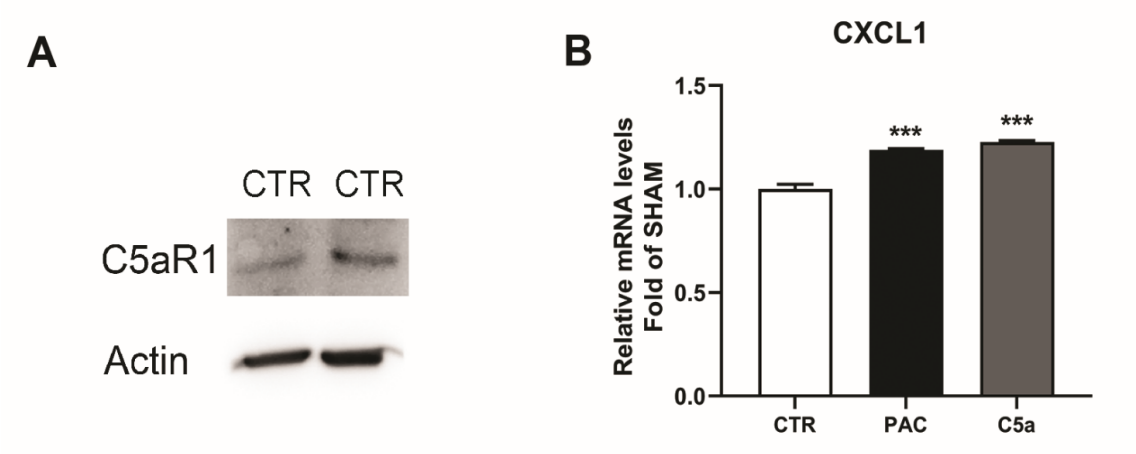


Figure 12: *A) C5aR1 expression in differentiated F11 cells; B) CXCL1 expression following paclitaxel or C5a treatment in F11 cells.*

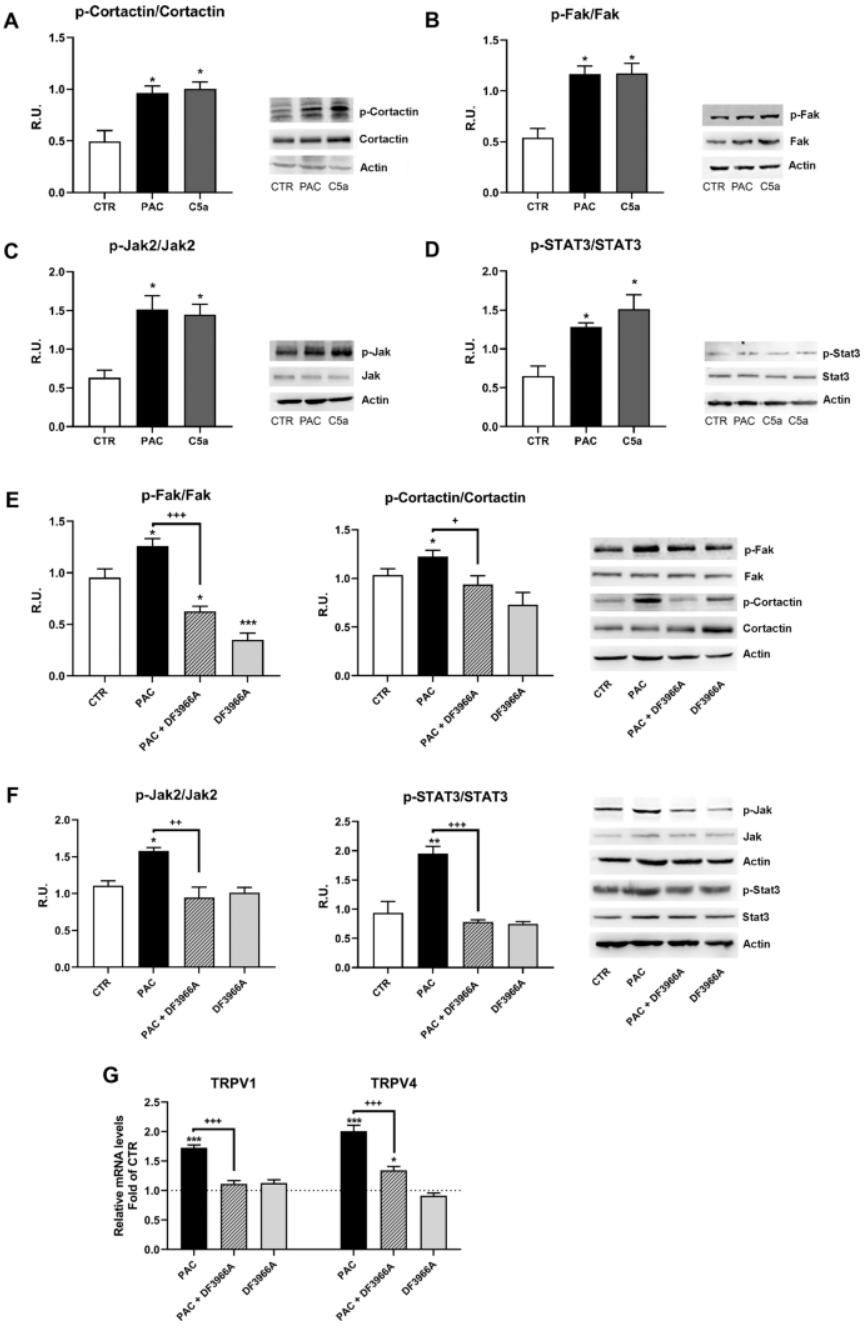


Figure 13: C5aR1 inhibition restores neuronal plasticity in paclitaxel-treated F11 cells. *Quantification of the expression of phosphorylated and total forms of p-cortactin (A), p-FAK (B), p-JAK2 (C) and p-STAT3 (D) in differentiated F11 cells following paclitaxel (PAC) or C5a treatment evaluated by western blot analysis. Expression of p-cortactin and p-FAK (E), p-JAK2 and p-STAT3 (F), and TRPV1 and TRPV4 (G) in differentiated F11 cells exposed to paclitaxel and treated or not with a C5aR1 inhibitor, DF3966A. F11 cells were treated for 24 h with PAC (10 nM), C5a (50 nM) or DF3966A (10 μM) or PAC (10 nM) + DF3966A (10 μM). ***P < 0.0005; **P < 0.005; *P < 0.05 vs CTR; +++P < 0.0005; ++P < 0.005; +P < 0.05 vs paclitaxel. Data are mean ± SEM of n = 3 different experiments.*

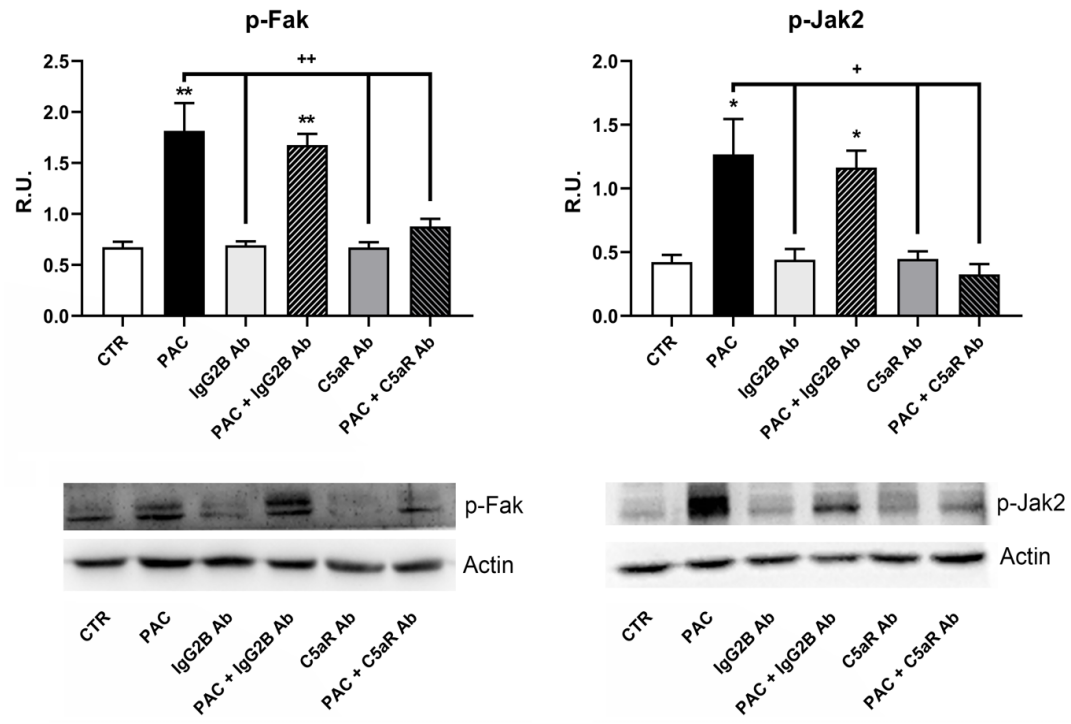


Figure 14: *Quantification of p-FAK and p-JAK2 expression in control (CTR), paclitaxel (PAC), IgG2B Antibody (Ab), PAC+ IgG2B Ab, C5aR Ab, PAC+C5aR Ab-treated F11 cells evaluated by western blot analysis. **P<0.005; *P<0.05 vs CTR; +P<0.05 ++ p<0.005 vs PAC. Data are mean ± SEM of n=3 different experiments.*

Additionally, C5aR1 inhibition fully restored the altered electrical activity of paclitaxel-treated DRG (**Figure 15**), confirming the pivotal role of C5aR1 in the neuropathological mechanisms of paclitaxel-induced CIPN. To explore the neuropathological mechanisms linked to paclitaxel-induced neurotoxicity and the involvement of C5aR1, electrophysiological analyses were performed on rat DRG primary cultures treated with paclitaxel (10 nM) or C5a (100 nM), with or without the C5aR1 inhibitor DF3966A (1 µM). Neurons exposed to paclitaxel displayed an immediate increase in action potential firing rates during short-term exposure (1 minute and 30 seconds). However, prolonged exposure (5 minutes) caused a significant reduction in electrical activity (-83.51% compared to control) due to voltage-gated channel inactivation (**Figures 15A, B, F**). Similarly, C5a rapidly elevated firing rates but led to a comparable reduction in neuronal electrical activity during extended exposure times (**Figures 15A-D, G**). These findings suggest that both paclitaxel and C5a induce rapid electrophysiological alterations in DRG neurons, highlighting shared mechanisms of action. The immediacy of these effects

supports the hypothesis that direct binding of paclitaxel or C5a to C5aR1 triggers these changes, rather than an indirect influence on the complement system. Administration of DF3966A effectively normalized the electrical activity disrupted by paclitaxel during both short and long exposures (**Figures 15C, F**). In addition, the inhibitor counteracted the C5a-induced increase in firing rates at short time points and significantly mitigated the reduction in activity (~35%) observed with prolonged exposure (**Figures 15E, G**). These results emphasize the pivotal role of C5aR1 in the development of paclitaxel-induced CIPN and demonstrate the protective potential of C5aR1 inhibition in preserving DRG neuronal function.

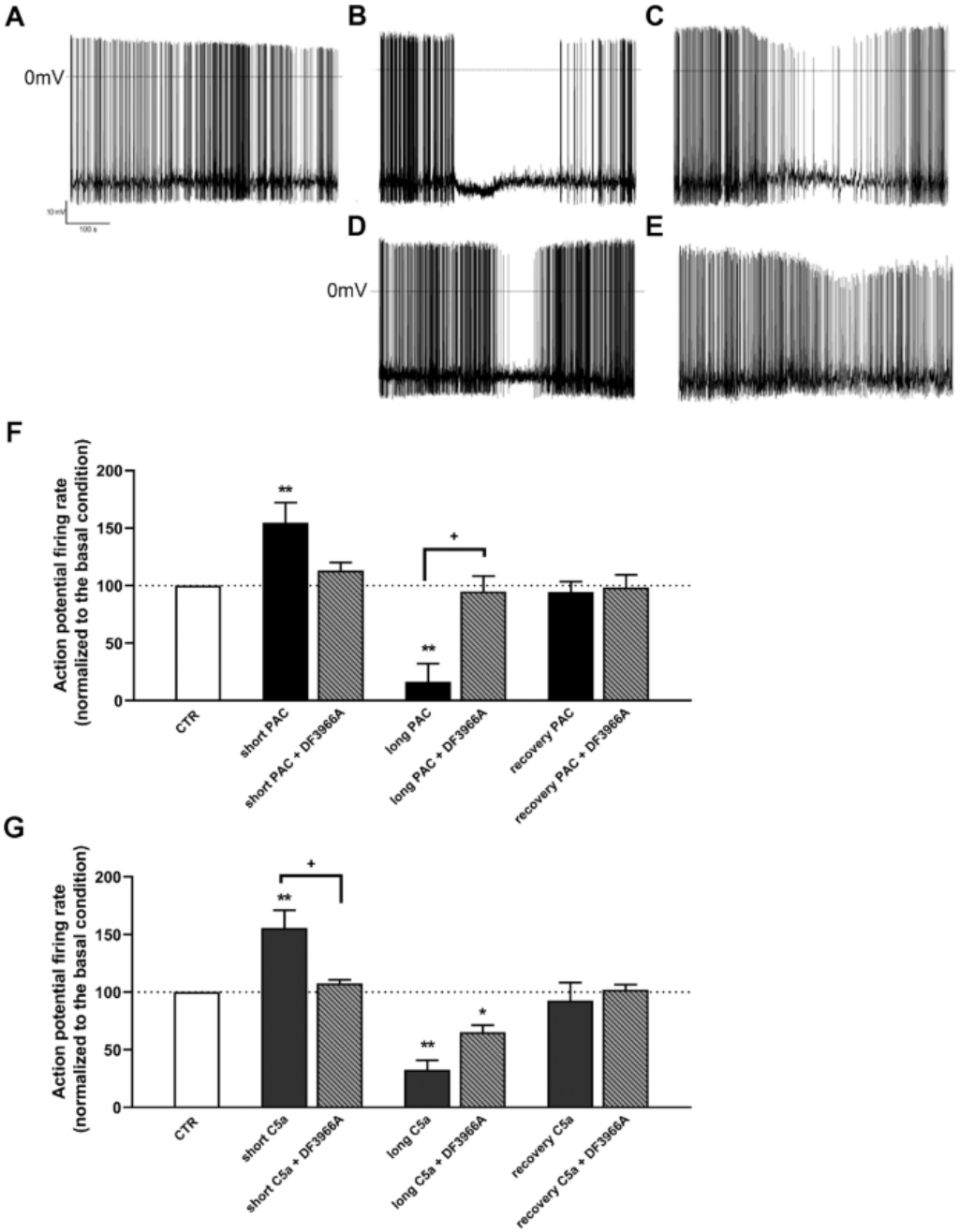


Figure 15: C5aR1 inhibition counteracts paclitaxel-induced alterations in rodent DRG. *Representative trace of electrophysiological recordings in DRG cells maintained under basal conditions (A), and in cells challenged with paclitaxel (10 nM) (PAC) (B), paclitaxel+DF3966A (1 μM) (C), C5a (100 nM) (D) or C5a + DF3966A (E). Quantitative evaluation of action potential firing rate in DRG exposed to paclitaxel alone or in combination with DF3966A (F), and C5a alone or in combination with DF3966A (G). DRG-derived neurons were cultured for 7 days. Cells were treated for 1 min 30 s (short) or 5 min (long). **P < 0.005 and *P < 0.05 vs respective basal condition; +P < 0.05 vs paclitaxel or C5a. Data are mean ± SEM of n = 3 different experiments.*

8.5 C5aR1 is a key mediator of paclitaxel-induced CIPN *in vivo*

To assess the involvement of C5aR1 in paclitaxel-induced CIPN *in vivo*, wild-type and C5aR1 knockout (KO) mice were administered paclitaxel intraperitoneally at 4 mg/kg on alternate days (days 1, 3, 5, and 7). Cold and mechanical allodynia symptoms were monitored following paclitaxel injections. As anticipated, wild-type mice developed cold allodynia from day 3 to day 14, in contrast to sham-treated mice (**Figure 16A**). Mechanical allodynia was also observed across all time points (days 1, 3, 5, 7, 10, and 14) in paclitaxel-treated mice compared to controls (**Figure 16B**). Importantly, C5aR1-deficient KO mice showed significant protection against both the induction and maintenance of paclitaxel-induced CIPN (**Figure 17**), highlighting the critical role of C5aR1 in the disease's pathophysiology. The involvement of paclitaxel and its vehicle, Cremophor EL (polyoxyethylated castor oil), in HSRs has been widely debated. While some studies implicate Cremophor EL, others highlight paclitaxel as the primary agent responsible. To clarify this, Balb/c mice were divided into four groups and treated with paclitaxel in Cremophor EL, paclitaxel in DMSO/PEG/TWEEN, or the vehicles alone. Mice exposed to paclitaxel in either formulation displayed pronounced HSRs, including increased vascular hyperpermeability and plasma extravasation, compared to vehicle-only groups (data not shown). These findings establish paclitaxel as the main inducer of HSRs, independent of its emulsifier.

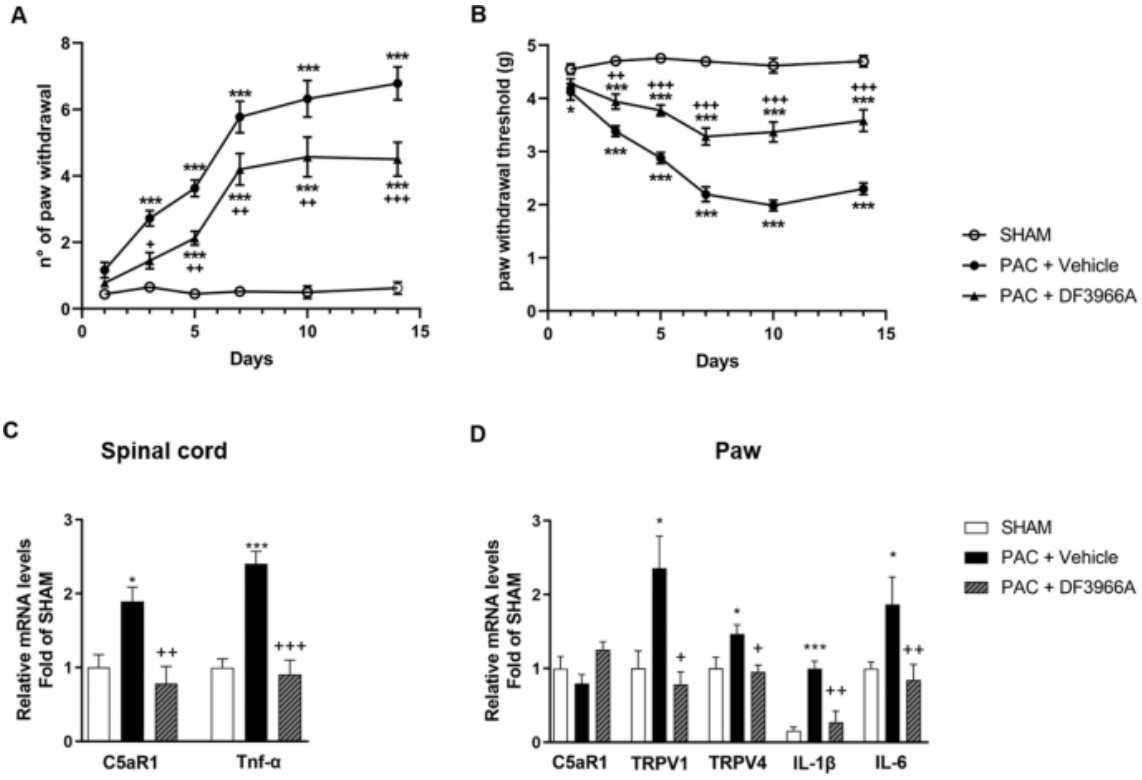


Figure 16: C5aR1 is a key mediator of paclitaxel-induced cold and mechanical allodynia. *Time course of paw withdrawal responses in mice displaying paclitaxel (PAC)-induced cold (A) (two-way ANOVA, effect of time × treatment F (10,285) = 21.69; p < 0.0001 followed by Bonferroni's multiple comparisons test) and mechanical (B) (two-way ANOVA, effect of time × treatment F (10,285) = 17.63; p < 0.0001 followed by Bonferroni's multiple comparisons test) allodynia treated or not with a C5aR1 inhibitor, DF3966A (30 mg/kg). DF3966A was orally administered every 12 h for 14 days during the induction phase of CIPN. Cold and mechanical allodynia were evaluated 3 h after DF3966A treatment on days 1, 3, 5, 7, 10 and 14. (C) Real time-PCR analysis of C5aR1 (one-way ANOVA, effect of treatment F (2,27) = 6.381; p = 0.0054 followed by Bonferroni's multiple comparisons test) and TNF-α (one-way ANOVA, effect of treatment F (2,27) = 14.79; p < 0.0001 followed by Bonferroni's multiple comparisons test) expression levels in the spinal cord, and of (D) C5aR1 (one-way ANOVA, effect of treatment F (2,27) = 3.480; p = 0.0452 followed by Bonferroni's multiple comparisons test), TRPV1 (one-way ANOVA, effect of treatment F (2,27) = 6.349; p = 0.0055 followed by Bonferroni's multiple comparisons test), TRPV4 (one-way ANOVA, effect of treatment F (2,27) = 4.050; p = 0.0290 followed by Bonferroni's multiple comparisons test), IL-1β (one-way ANOVA, effect of treatment F (2,27) = 13.81; p < 0.0001 followed by Bonferroni's multiple comparisons test) and IL-*

*6 (one-way ANOVA, effect of treatment F (2,27) = 1.898; p = 0.1694 followed by Bonferroni's multiple comparisons test) in paw samples collected at day 15 after the first paclitaxel administration. ***P < 0.0005 and *P < 0.05 vs respective sham group; +++P < 0.0005; ++P < 0.005 and +P < 0.05 vs paclitaxel. Data are expressed as mean ± SEM; n = 10 per group.*

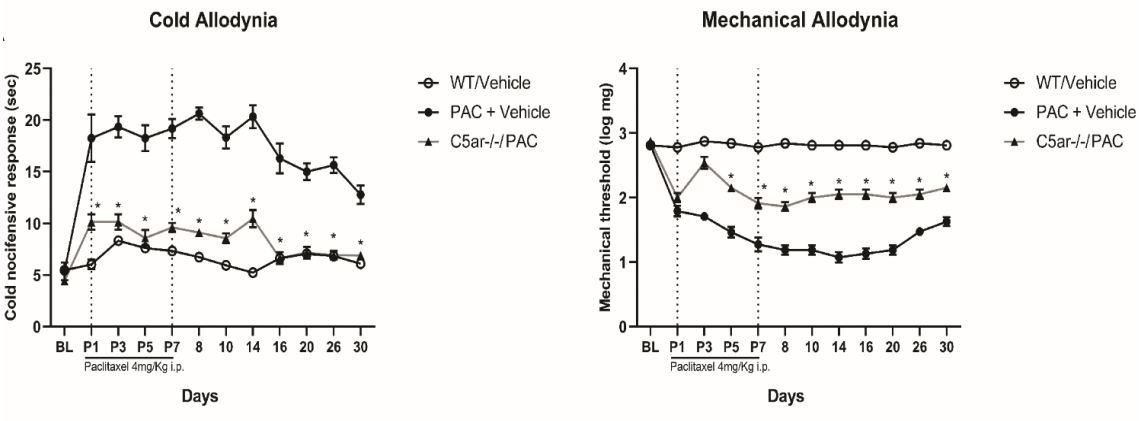


Figure 17: Cold and mechanical allodynia in C5ar1$^{-/-}$ mice. *Time course of the effect of paclitaxel (PAC) on cold (A) and mechanical (B) allodynia in wild-type (WT) and C5ar1$^{-/-}$ mice. *P<0.05 vs WT. Data are shown as mean ± SEM of 10 animals per group.*

Building upon these findings, the therapeutic potential of C5aR1 inhibition was assessed. The pharmacological inhibition of C5aR1 using oral DF3966A at a dose of 30 mg/kg, administered every 12 hours for 14 days during the CIPN induction phase, notably reduced cold allodynia induced by paclitaxel on days 3, 5, 7, 10, and 14 (**Figure 16A**). Additionally, mechanical allodynia caused by paclitaxel was significantly inhibited from day 3 to day 14 (**Figure 16B**). These results highlight the crucial role of C5aR1 in paclitaxel-induced CIPN and suggest that targeting C5aR1 could offer a potential therapeutic strategy for this condition.

To explore the underlying mechanisms of C5aR1 inhibition's protective effects in paclitaxel-induced CIPN, gene expression of C5aR1, inflammatory markers, and TRPV1/4 was assessed via real-time PCR in spinal cord and paw samples collected from mice on day 15 post-paclitaxel administration. In the spinal cords of paclitaxel-treated mice, C5aR1 expression was notably elevated compared to control animals, and this upregulation was fully reversed by DF3966A treatment (**Figure 16C**). Additionally,

DF3966A significantly reduced paclitaxel-induced TNF-α mRNA expression, a key mediator of spinal microglial activation and neuropathic pain (**Figure 16C**). In contrast, paclitaxel alone or in combination with DF3966A did not alter C5aR1 mRNA levels in paw samples. However, paclitaxel treatment increased TRPV1, TRPV4, IL-1β, and IL-6 mRNA levels, while DF3966A administration led to a marked reduction in these levels (**Figure 16D**). These results indicate that spinal C5aR1 activation by paclitaxel is crucial for modulating peripheral inflammatory cytokines, which play a role in the enhancement of pain signaling.

8.6 C5aR1 inhibition counteracts paclitaxel- or C5a-induced anaphylactoid cytokine release in macrophages

The potential role of C5aR1 in paclitaxel-induced anaphylaxis was explored, given the involvement of the C5a-C5aR1 axis in anaphylactoid responses. Macrophages, which express C5aR1 and are known to be pivotal in CIPN and anaphylaxis, were utilized to examine this hypothesis. Mouse (RAW 264.7) and human (THP-1) macrophages were exposed to paclitaxel (10 nM) or C5a (10 nM) for 24 hours, followed by cytokine profiling using a proteome profiler assay. Both paclitaxel and C5a treatments induced cytokine profiles resembling typical anaphylaxis, with the only distinction being the release of C5a, which occurred exclusively in C5a-stimulated cells (**Figure 18**). In paclitaxel-treated RAW 264.7 cells, increased levels of MCP-1, a chemokine involved in anaphylaxis and basophil migration, along with MIP-1α, MIP-1β, MIP-2, and IL1Ra, were observed (**Figures 18A, B**). C5aR1 inhibition using Avacopan (1 μM), anti-C5aR1 antibody (1 μM), or DF3966A (1 μM) effectively suppressed cytokine release, reinforcing the involvement of C5aR1 in mediating paclitaxel-induced anaphylactoid reactions (**Figures 18A, B**).

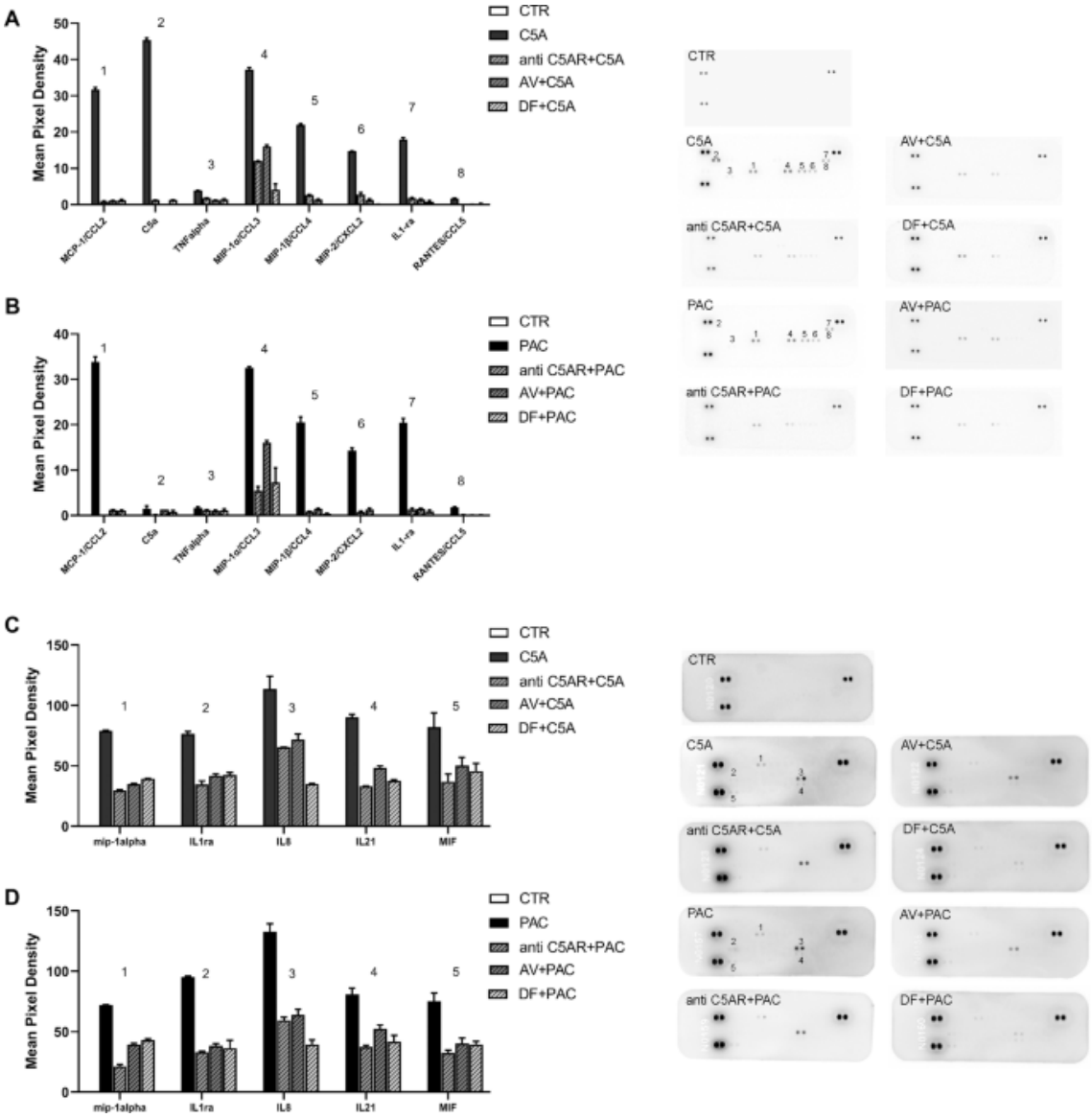


Figure 18: C5aR1 inhibition blocks C5a- and paclitaxel-induced anaphylactoid cytokine release from mouse and human macrophages. *Proteome profiles of cytokines released from RAW 264.7 (A, B) and human (C, D) macrophages challenged with C5a (A, C) or paclitaxel (B, D) alone or in combination with a C5AR1 antibody (anti C5AR), Avacopan or DF3966A. RAW 264.7 and human macrophages cells were treated with C5a (10 nM), or paclitaxel (PAC) (10 nM), or DF3966A (1 μM) or Avacopan (1 μM) and C5aR1 antibody (1 μM) for 24 h. Data are mean ± SEM of n = 3 different experiments.*

The analysis conducted on human macrophages corroborated the findings from murine models, showing that the cytokine profile induced by C5a was similar to that triggered by paclitaxel. In these conditions, the use of DF3966A, Avacopan, and a C5aR1 antagonist effectively inhibited the cytokine release caused by both C5a and paclitaxel

(**Figures 18C, D**). Additionally, both agents stimulated IL-8 release from human macrophages, which was blocked by Avacopan, anti-C5aR1 antibody, and DF3966A (**Figures 18C, D**). These results suggest that C5aR1 might mediate paclitaxel-induced anaphylactoid reactions, prompting further *in vivo* investigation.

8.7 C5aR1 inhibition prevents paclitaxel-induced anaphylactoid reactions *in vivo*

After identifying paclitaxel as the primary cause of chemotherapy-induced anaphylactoid reactions (**Figure 17**), the potential of C5aR1 inhibition as a preventive measure for paclitaxel-induced hypersensitivity reactions (HSRs) was evaluated. Inhibition of C5aR1 was achieved through pre-treatment with either DF3966A (30 mg/kg, oral) or Avacopan (30 mg/kg, oral), administered 1–3 hours prior to paclitaxel based on their pharmacokinetics (data not shows). Both inhibitors effectively prevented the onset of HSRs, eliminating paclitaxel-induced vascular leakage and plasma extravasation (**Figures 19A, B**). Furthermore, pre-treatment with these agents notably reduced serum levels of histamine and complement 5b-9 (SC5b-9) (**Figures 19C, D**), which are linked to complement pathway activation. These findings highlight the pivotal role of C5aR1 in the initial inflammatory phase of paclitaxel-induced HSRs, suggesting that its inhibition may be a promising strategy to prevent paclitaxel-induced anaphylaxis.

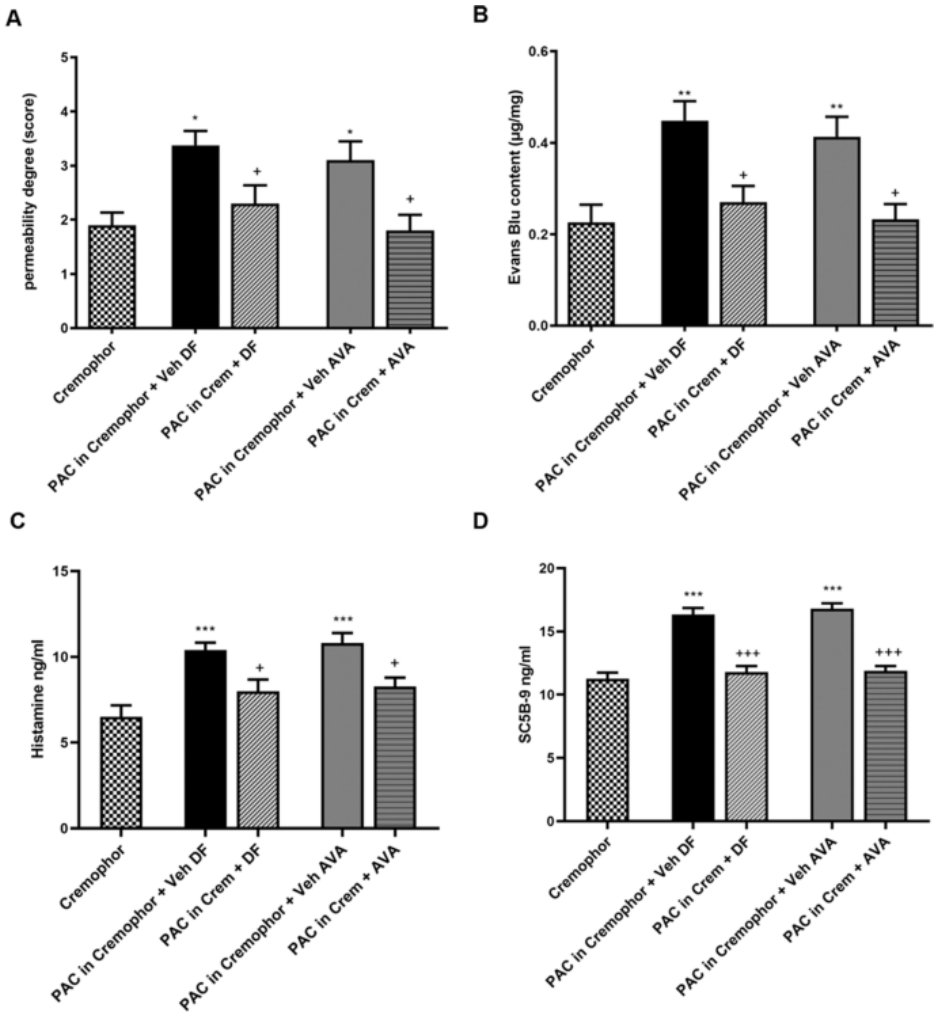


Figure 19: C5aR1 inhibition prevents anaphylactoid reactions induced by paclitaxel in mice. *Effect of vehicle, DF3966A or Avacopan on paclitaxel-increased vascular permeability (A) (one-way ANOVA, effect of treatment F (4, 44) = 5.121; p = 0.0018 followed by Bonferroni's multiple comparisons test) and plasma extravasation (B) (one-way ANOVA, effect of treatment F (4,44) = 7.258; p = 0.0001 followed by Bonferroni's multiple comparisons test) in mice. Serum histamine (one-way ANOVA, effect of treatment F (3,36) = 7.590; p = 0.0005 followed by Bonferroni's multiple comparisons test) (C) and SC5B-9 (one-way ANOVA, effect of treatment F (3,36) = 24.74; p < 0.0001 followed by Bonferroni's multiple comparisons test) (D) concentration in mice receiving paclitaxel pre-treated with vehicle, DF3966A or Avacopan. Paclitaxel was intravenously administered at 20 mg/kg. Vehicle DF3966A (saline) and DF3966A (30 mg/kg, os) were administered 3 h before paclitaxel, while vehicle Avacopan and Avacopan (30 mg/kg, os) were administered 1 h before paclitaxel. ***P < 0.0005, **P < 0.005 and *P < 0.05 vs Cremophor group; +++P < 0.0005; ++P < 0.005 and +P < 0.05 vs paclitaxel. Data are shown as mean ± SEM of 10 animals per group.*

CHAPTER IX

9.1 Discussion

Paclitaxel, widely employed in cancer therapy, is frequently associated with adverse effects such as CIPN and HSRs, both of which significantly compromise patients' quality of life. This study identifies C5aR1 as a previously unrecognized target of paclitaxel, demonstrating its pivotal role in mediating both CIPN and anaphylactoid reactions. Additionally, it provides evidence that C5aR1 inhibition effectively counteracts these adverse effects *in vitro* and *in vivo*. Numerous prior studies have highlighted paclitaxel's pro-inflammatory properties, specifically its ability to induce IL-8 expression, which is strongly implicated in the onset and progression of neuropathy [443,446]. Utilizing the supercomputing platform EXaSCale smArt pLatform Against paThogEns (EnsEXSCALATE), this investigation aimed to identify upstream mediators of IL-8 potentially binding to paclitaxel and contributing to its side effects [447]. Unexpectedly, C5aR1 was identified as one of the receptors exhibiting the highest predicted affinity for paclitaxel, a finding later confirmed through SPR and competition experiments. The activation of C5aR1 by paclitaxel triggers downstream pathways, including NF-kB/P38 and c-Fos/c-Jun, both of which regulate IL-8 expression and are implicated in neurotoxicity [441]. Subsequent experiments demonstrated that C5aR1 activation in sensory neurons (F11 cells) resulted in alterations in key signaling pathways, including p-FAK, p-cortactin, and JAK-STAT. The involvement of CXCL1 in sustaining and exacerbating these changes was evidenced through phosphorylation time-point studies. Crucially, these neurotoxic effects were mitigated by C5aR1 inhibition, using either a specific antibody (C5aR1 Ab) or a receptor antagonist, further corroborating C5aR1's role in mediating paclitaxel-induced neuropathy. The complement system, long recognized for its role in inflammation and pain modulation, has not been thoroughly investigated in the context of CIPN. Using C5aR1 knockout mice and pharmacological inhibitors, this study demonstrated a significant reduction in paclitaxel-induced allodynia, confirming the receptor's critical role in neuropathic pain. Additionally, electrophysiological recordings from DRG neurons revealed that both paclitaxel and C5a produced comparable alterations, which were completely reversed by C5aR1 inhibition. These findings strongly suggest that C5aR1 is essential in the development of paclitaxel-induced CIPN and that its blockade represents a promising strategy for prevention or treatment. Recent research has also implicated the complement system, specifically

component 3 (C3), in CIPN. Reduced mechanical allodynia observed in C3 knockout rats aligns with the current findings, further supporting the hypothesis that complement components are viable therapeutic targets [408]. Existing therapeutic options for CIPN are limited, focusing primarily on symptom management rather than prevention or reversal of the underlying pathology. The standard approaches, such as the use of gabapentinoids, antidepressants, or topical agents, provide only partial relief, leaving many patients with persistent neuropathic symptoms that severely impair their quality of life. Furthermore, these therapies often come with their own adverse effects, which may complicate treatment regimens for cancer patients already burdened by polypharmacy. Current strategies fail to address the molecular and cellular mechanisms driving CIPN [15]. Building upon this framework, the present study establishes that (i) paclitaxel directly binds to and activates C5aR1, (ii) C5aR1 is a critical mediator of CIPN, and (iii) its inhibition effectively prevents or mitigates this condition. Given the limited treatment options for established CIPN, targeting C5aR1 represents a novel and preventive pharmacological approach. The involvement of the complement system extends beyond neuropathy to include immune-mediated hypersensitivity. C5a, a potent anaphylatoxin, and its receptor, C5aR1, are well-established contributors to asthma and antigen-induced anaphylaxis [448,449]. This study hypothesized that C5aR1 might also mediate paclitaxel-induced HSRs and anaphylaxis. Supporting this hypothesis, macrophage models (RAW 264.7 and THP-1) exhibited overlapping proteomic profiles when stimulated with paclitaxel or C5a. Cytokine release induced by both treatments was effectively suppressed by C5aR1 inhibition. The inclusion of THP-1 cells allowed for the evaluation of NLRP3 inflammasome involvement, which plays a pivotal role in immune responses and interacts with the complement pathway. *In vivo* experiments confirmed that paclitaxel, rather than its solvent Cremophor EL, is primarily responsible for inducing anaphylactoid reactions during chemotherapy. The central role of C5aR1 in paclitaxel-induced HSRs was established, and its inhibition emerged as a potential therapeutic strategy for preventing such adverse effects. These findings are particularly relevant given that other taxanes, including docetaxel and next-generation formulations like nab-paclitaxel, are similarly associated with CIPN and HSRs [450]. The interplay between the complement system and cancer biology has gained increasing attention, with multiple studies highlighting the C5a-C5aR1 axis in various malignancies. For instance, C5a deficiency or C5aR1 blockade has been shown to reduce tumor growth and enhance chemotherapy efficacy in colorectal and lung cancer models [451,452]. Furthermore,

preclinical studies suggest synergistic benefits when complement inhibitors are combined with immune checkpoint inhibitors, potentially through reduced recruitment and polarization of myeloid cells [452]. The combination of paclitaxel with C5aR1 inhibitors may therefore offer dual benefits: preventing CIPN and HSRs while amplifying anti-tumor effects. Immunotherapy has already been explored in combination with paclitaxel for treating various cancers, yielding promising outcomes [453]. Given the demonstrated efficacy of complement inhibitors in modulating immune responses and enhancing chemotherapy, further investigations into their combined use with paclitaxel are warranted. This dual approach may enhance treatment efficacy by addressing both the oncological and neuropathic dimensions of paclitaxel use. The potential for combining complement inhibitors with immune checkpoint inhibitors further expands the therapeutic landscape, offering synergistic benefits in cancer treatment. Moreover, advances in computational drug discovery platforms, such as EnsEXSCALATE, provide powerful tools for identifying novel upstream mediators of CIPN. These platforms can accelerate the discovery of new drug candidates and optimize their specificity and efficacy, reducing the time required to bring these innovations to clinical practice. Additionally, the development of predictive biomarkers for CIPN risk, such as genetic or proteomic markers associated with C5aR1 signaling, could enable personalized treatment strategies. Early identification of at-risk patients would allow for preemptive interventions, significantly improving outcomes.

In summary, advancing the understanding of CIPN pathophysiology, with a particular focus on the pivotal role of C5aR1, provides a promising therapeutic avenue. Targeting C5aR1 offers the potential to transform CIPN management from merely alleviating symptoms to achieving both prevention and treatment. A shift toward mechanism-based therapies, guided by molecular insights such as C5aR1 involvement, is urgently needed to address these limitations and improve patient outcomes effectively. Future research should prioritize large-scale clinical trials to validate these preclinical findings and explore predictive biomarkers for CIPN. Advances in computational drug discovery platforms, such as EnsEXSCALATE, offer promising avenues for the identification of selective inhibitors. Furthermore, integrating C5aR1 blockade with immune checkpoint inhibition may provide a dual therapeutic benefit by enhancing anti-tumor responses. Future studies will ultimately refine these therapeutic approaches further.

CHAPTER X

10.1 Conclusions

In conclusion, C5aR1 is a new target of paclitaxel, playing a crucial role in the mediation of paclitaxel-induced CIPN and HSRs. Inhibition of C5aR1 was shown to effectively counteract these side effects. These findings are pivotal, offering a better understanding of the mechanisms involved in the pathophysiology of CIPN and HSRs related to paclitaxel treatment. Furthermore, C5aR1 emerges as a potential pharmacological target for preventing such adverse reactions. This breakthrough opens new possibilities for managing the growing number of patients facing severe long-term consequences or even death after paclitaxel therapy. Recent literature also described the involvement of C5aR1 in other neurological condition such as Amyotrophic Lateral Sclerosis, Huntington's disease, Alzheimer's disease and Parkinson's disease, further supporting the importance of C5aR1 molecular exploration for a broadened therapeutic landscape.

CHAPTER XI

11.1 Bibliography

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Tesi redatta con il sostegno finanziario del programma

FSE-REACT-EU, PON “Ricerca e Innovazione” 2014-2020 (D.M. 1061/2021)

Asse IV “Istruzione e ricerca per il recupero – REACT-EU”

Azione IV.4 “Dottorati e Contratti di ricerca su tematiche dell'innovazione”



