Programa de Doctorado en Biología Molecular y Celular

INVESTIGATING CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHIES AND ITS SEXUAL DIMORPHISM USING AN *IN VITRO* PRECLINICAL PAIN MODEL

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INFORMA:

Que Dña. *Eva María Villalba Riquelme* ha realizado bajo mi supervisión el trabajo titulado **"**INVESTIGATING CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHIES AND ITS SEXUAL DIMORPHISM USING AN *IN VITRO* PRECLINICAL PAIN MODEL**"** conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Y para que así conste a los efectos oportunos, se expide por escrito.

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INFORMA:

Que Dña. *Eva María Villalba Riquelme* ha realizado bajo la supervisión de nuestro Programa de Doctorado el trabajo titulado **"**INVESTIGATING CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHIES AND ITS SEXUAL DIMORPHISM USING AN *IN VITRO* PRECLINICAL PAIN MODEL" conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

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todo lo bueno que pueda haber hecho y haya en esta tesis,

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Eres el mejor Abuelo del mundo.

Te quiero muchísimo Abuelo.

Te envío un abrazo muy muy fuerte y muchos besos al cielo.

Index

List of Abbreviations

AHP	Afterhyperpolarization phase
AITC	Allyl isothiocyanate
AP	Action potential
ASIC	Acid sensing ion channels
Caps	Capsaicin
Ca_V	Voltage-gated calcium channels
CGRP	Calcitonin-gene related peptide
CIPN	Chemotherapy-Induced Peripheral Neuropathy
DIV	Days *in vitro*
DRG	Dorsal root ganglion
G-V	Conductance – Voltage relationship
HCN	Hyperpolarization-activated cyclic nucleotide-gated channel
hDRG	Human dorsal root ganglion
IASP	International Association for the Study of Pain
IB4	Isolectin B4
IENF	Intra-epidermal nerve fibre
iPSC	Induced pluripotent stem-cells
IQR	Interquartile range
J-V	Current density – Voltage relationship
K2P	Two-pore K^+ channel
K_A	Fast-inactivating potassium current
K_{DR}	Delayed rectifier potassium current
Kir	Inward rectifier channels
K_V	Voltage-gated potassium channels
MEA	Microelectrode/multielectrode array
Na_V	Voltage-gated sodium channels
OIPN	Oxaliplatin-induced peripheral neuropathy

PIPN	Paclitaxel-induced peripheral neuropathy
RMP	Resting membrane potential
Rpl29	Ribosomal protein L29
SA	Spontaneous activity
tAHP	Time to recover from the AHP
TG	Trigeminal ganglia
TLR	Toll-like receptor
tpeak	Time to reach the maximum voltage of the AP
tr	Repolarization time
TREK	TWIK-1 related K^+ channel
TRAAK	TWIK-related arachidonic acid activated K^+ channel
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPM3	Transient Receptor Potential Melastatin 3
TRPM8	Transient Receptor Potential Melastatin 8
TRPV1	Transient receptor potential vanilloid 1
TRPV2	Transient receptor potential vanilloid 2
TRPV4	Transient receptor potential vanilloid 4
TTX	Tetrodotoxin
Vth	Voltage threshold

Resumen/Abstract

Resumen

El **dolor crónico** es uno de los problemas de salud con mayor incidencia en la sociedad actual. En el caso de los pacientes con cáncer, el dolor producido por los tratamientos quimioterapéuticos es tan severo que se considera uno de los principales efectos adversos por el cual deben reducir o incluso interrumpir su tratamiento, comprometiendo la supervivencia y la calidad de vida de estos pacientes. Este dolor se desencadena como consecuencia de la neuropatía periférica inducida por la quimioterapia (**CIPN**). Gracias a numerosos estudios realizados a nivel preclínico y clínico, se sabe que el origen de estas neuropatías se debe a la alteración que generan los tratamientos quimioterapéuticos sobre la actividad de las neuronas del sistema nervioso periférico encargadas del envío de señales nocivas, conocidas como **nociceptores**. Sin embargo, se desconocen los mecanismos exactos que alteran la funcionalidad en estos nociceptores desencadenando la generación y mantenimiento de este dolor. Debido a esta falta de conocimiento, actualmente no existe ningún tratamiento efectivo aprobado para aliviar estos síntomas. Ante esta situación, se diseñó la presente tesis doctoral con el objetivo de investigar los mecanismos funcionales y moleculares implicados en la sensibilización producida por dos de los quimioterapéuticos prescritos con mayor frecuencia: paclitaxel y oxaliplatino. El primer objetivo consistió en desarrollar un modelo preclínico con el que poder estudiar exposiciones prolongadas a quimioterapéuticos y estudiar el curso temporal de sus efectos neurotóxicos. Para ello, se estableció y caracterizó un cultivo celular *in vitro* con viabilidad de hasta 10 días a partir de DRG provenientes de ratas adultas. Posteriormente, se investigó el efecto de la exposición directa del paclitaxel y oxaliplatino sobre los nociceptores en cultivo. Usando este modelo de neuropatía inducida por **paclitaxel**, observamos un incremento en la excitabilidad de los nociceptores alcanzando un máximo de actividad a las 48 h después del tratamiento, el cual se revierte en ausencia prolongada del quimioterapéutico. Estos datos reproducen los resultados publicados previamente en estudios clínicos y muestran el potencial traslacional de este modelo para el estudio de estas neuropatías. Gracias a estos cultivos longevos de DRG, pudimos observar que el aumento de la actividad se produjo tanto en las neuronas IB4(-) como IB4(+). Además, este se correlacionó con una mayor funcionalidad de los canales iónicos **$Na_V1.8$**, **TRPV1** y **TRPM8**, postulando estos canales iónicos como potenciales dianas terapéuticas para tratar la CIPN. Sin embargo, cuando analizamos los niveles de ARNm de estos canales, no se detectaron diferencias significativas, indicando que la desregulación de estos canales estaría ocurriendo mediante mecanismos postraduccionales en vez de postranscripcionales. Además, debido al creciente número de evidencias sobre la existencia de **dimorfismo sexual** en la sintomatología y las vías fisiopatológicas del dolor, se estableció como objetivo a su vez analizar estas posibles diferencias examinando por separado cultivos obtenidos de ratas macho y hembra. Con esta

segregación, pudimos encontrar que las hembras eran más sensibles al efecto del paclitaxel, encontrando un aumento más pronunciado en la excitabilidad de sus nociceptores tras el tratamiento.

En el caso del modelo de neuropatía inducida por **oxaliplatino**, se observó un efecto dependiente del tiempo de exposición, en el cual duraciones de exposición más largas (48 h comparado con 24 h) produjeron la sensibilización de las neuronas. Inmediatamente después del tratamiento de 48 h, se observó un aumento de la excitabilidad de las neuronas. Este aumento estaba producido principalmente por el subtipo neuronal IB4(+), en el que se encontró una reducción significativa en la reobase y un aumento de la frecuencia de disparo de potenciales de acción. En relación con este efecto, pudimos observar una alteración en la cinética de los canales iónicos **Na_V**, enlenteciendo su inactivación y agilizando la recuperación frente a la inactivación. Analizando la corriente mediada por los canales iónicos TRP, encontramos un aumento en las corrientes mediadas por **TRPV1** y **TRPA1**, mientras que las producidas por activación del canal iónico TRPM8 se mantuvieron inalteradas. Mediante PCR cuantitativa detectamos que el oxaliplatino producía un aumento en la expresión del ARNm de TRPV1 y TRPA1, sugiriendo una sobreexpresión de estos canales iónicos en las neuronas como posible mecanismo para explicar el incremento en la excitabilidad de las IB4(+). Sin embargo, estas respuestas al tratamiento con oxaliplatino no ocurrieron igual en los cultivos procedentes de ambos sexos. De hecho, el oxaliplatino sólo despolarizó el potencial de membrana de las neuronas extraídas de cultivos de ratas **hembra**. Además, se encontraron diferencias significativas en los mecanismos moleculares subyacentes. Las hembras presentaron una reducción de la densidad de corriente mediada por los canales K_V y un aumento de la producida por los canales Na_V, mientras que los **machos** presentaron una mayor duración de la corriente de los Na_V y una recuperación más rápida tras su inactivación, pero menor densidad de corriente mediada por estos canales. A su vez, los machos presentaron un porcentaje mayor de neuronas que respondieron al agonista específico de TRPA1, sugiriendo una mayor desregulación en los niveles del canal TRPA1 en este sexo inmediatamente después del tratamiento con oxaliplatino.

De esta forma, los datos obtenidos con ambos modelos sugerían que el dimorfismo sexual podría tener una gran influencia en la CIPN. Además, los canales TRP parecían jugar un papel relevante en estas diferencias. Para explorar este tema con mayor profundidad, realizamos una revisión sobre los estudios que abordaron el papel de los canales termoTRP en machos y hembras con dolor crónico con un fuerte componente periférico: migraña crónica y CIPN. Una de las conclusiones de este trabajo fue la necesidad de incorporar y segregar ambos sexos en los ensayos clínicos y preclínicos, ya que en muchos de ellos no se analizaron por separado y, cada vez, más evidencias apuntan hacia la presencia de dimorfismo sexual en las vías de señalización del dolor. Valorando las opciones terapéuticas, destacamos la posibilidad de modular estos canales mediante el desarrollo de formulaciones tópicas

adaptadas al sexo del individuo, ya que permitirían minimizar la aparición de posibles efectos adversos. Pero, para lograr la eficacia de estos tratamientos en toda la población, surge la necesidad de seguir investigando para comprender los mecanismos moleculares que expliquen este dimorfismo sexual. Sólo a través de este conocimiento se podrán desarrollar y adaptar los fármacos para que sean más efectivos en hombres y mujeres, consiguiendo un tratamiento satisfactorio y personalizado frente al dolor crónico.

En conclusión, en esta tesis doctoral se han desarrollado dos modelos *in vitro* preclínicos que emulan la neuropatía inducida por los agentes quimioterapéuticos paclitaxel y oxaliplatino. Con estos modelos, hemos podido replicar resultados publicados anteriormente en ensayos clínicos mostrando su potencial para estudiar las neuropatías periféricas. Además, hemos podido identificar posibles dianas terapéuticas para tratar el dolor crónico producido por el tratamiento con oxaliplatino y paclitaxel. Por último, esta tesis muestra la novedosa presencia de dimorfismo sexual en la respuesta a estos agentes y abre una nueva vía para realizar tratamientos dirigidos frente a la CIPN.

Abstract

Chronic pain is one of the health problems with a stronger incidence in society. In the case of cancer patients, the pain evoked by chemotherapeutic agents can be so severe that it is considered one of the major adverse effects by which they have to reduce or even stop their treatment, compromising the survival and quality of life of these patients. This pain evolves as a consequence of the peripheral neuropathy induced by the chemotherapy (**CIPN**). Thanks to numerous studies performed at clinical and preclinical levels, it is well-known that this neuropathy is produced by the alteration of the activity of the neurons of the peripheral nervous system responsible of sending noxious signals, known as nociceptors. Nevertheless, the exact mechanisms underlying the generation and maintenance of the pain symptoms remain unknown. Due to this lack of knowledge, nowadays there is no effective treatment approved for alleviating this pain. To address this issue, the present doctoral thesis was designed with the aim of investigating the functional and molecular mechanisms implicated in the sensitization produced by two of the most common chemotherapeutic agents: paclitaxel and oxaliplatin.

The first objective consisted of developing a preclinical *in vitro* model that allowed to investigate prolonged exposures of sensory neurons to chemotherapeutic agents and studying the time course of the algesic effect. For this task, we established and characterized a long-term cell culture of rat DRG nociceptors that could survive up to 10 days *in vitro*. Then, the effects of the direct exposure to paclitaxel and oxaliplatin on the cultured nociceptors were investigated. As a result of the **model of paclitaxel-induced neuropathy**, we observed a reversible sensitization in the activity of the neurons that peaked 48 h after eliminating the treatment, virtually dissipating following prolonged absence of the chemotherapeutic. These data reproduced the results published in previous clinical trials, suggesting the translational potential of the model. Thanks to these long-term cultures, we could observe that the increase in the activity was present in both IB4(-) and IB4(+) neurons. In addition, this effect was correlated with a higher activity of the ion channels TRPV1, TRPM8, and $Na_V1.8$, emerging as potential therapeutic targets to treat the CIPN. However, when we analyzed the mRNA levels of these channels, we could not detect significant differences, indicating that the channels dysregulation was occurring by post-translational rather than transcriptional mechanisms. Besides, due to the cumulative evidence on the existence of sexual dimorphism in the symptomatology and physiopathological pathways of pain, an additional objective was established to analyze the possible sex differences examining separately the cultures obtained from male and female rats. With this segregation, we could find that females were more sensitive to paclitaxel, showing a more pronounced increase in the nociceptors' excitability after its treatment.

For the **model of oxaliplatin-induced neuropathy**, oxaliplatin produced a time-dependent effect, with longer incubation times (48 h compared with 24 h exposure) driving to a higher sensitization of the neurons. Immediately after the 48 h treatment, the sensory neurons exhibited a notorious increase in the excitability. This increase was mainly produced in the IB4(+) neuronal subtype, where we could detect a reduced rheobase and a higher firing frequency of action potentials. Related to this effect, we observed an alteration in the kinetics of the Na_V channels, slowing down their inactivation and promoting the recovery from their fast inactivation. Analyzing TRP channels, enhanced activity of TRPV1 and TRPA1 channels was detected without affecting TRPM8 functionality. As a possible explanatory mechanism, using quantitative PCR, we found higher TRPV1 and TRPA1 mRNA levels, suggesting that oxaliplatin could modify these channels by transcriptional mechanisms. Interestingly, the response to oxaliplatin showed a sexual dimorphic effect. Oxaliplatin only depolarized the resting membrane potential of the neurons extracted from female rats. Moreover, significant sex differences were found in the underlying molecular mechanisms. Females showed increased Na_V and diminished K_V current density, whereas males presented bigger expression of the TRPA1 ion channel and longer duration and faster recovery of the Na_V current.

In this way, the data obtained with both models suggested that the sexual dimorphism could have a strong influence in CIPN. Besides, TRP channels seemed to play a pivotal role in these differences. To further explore this topic, we conducted a review on the role of thermoTRP channels in males and females in two types of chronic pain with a strong peripheral component: chronic migraine and CIPN. One of the conclusions that we could extract from this work was the need of incorporating and segregating both sexes in clinical and preclinical assays, because most of these studies did not analyze them separately and, nowadays, an increasing number of evidence points towards the presence of sexual dimorphism in pain pathways. Studying the different therapeutic options available, we remarked the possibility of modulating these channels with topical formulations, due to the lower associated adverse effects. However, to achieve the effectiveness of these treatments in the whole population, there is a need of continue investigating the molecular details that could explain this sexual dimorphism. Only from this knowledge we could develop and adapt more effective pharmacological drugs for men and women, reaching a satisfactory treatment against chronic pain.

Taken together, in this doctoral thesis we have developed two *in vitro* preclinical models that emulated the neuropathy induced by the chemotherapeutic agents' paclitaxel and oxaliplatin. With these *in vitro* models, we could replicate previous data obtained in clinical and preclinical assays, showing its translational potential for investigating the peripheral neuropathies. Besides, we could identify potential therapeutic targets for treating the chronic pain produced by paclitaxel and oxaliplatin treatments. Finally, the present doctoral thesis shows the novel presence of sexual

dimorphism in response to these agents opening a new venue to develop personalized treatments against CIPN.

Introduction

Introduction

Pain

From the origin of humanity, pain has been present in our lives. Defining pain has been a major challenge for the scientific community but according to the last upgrade provided by the International Association for the Study of Pain (IASP) in 2020, pain can be defined as: "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (IASP, 2020).

Despite representing an unpleasant sensation, pain has been considered a vital mechanism for survival. It constitutes a crucial alert system that triggers protective responses to minimize the organism damage and guarantee its wellbeing, like running away from a flame or guarding a broken arm to allow healing (Basbaum et al., 2009). Indeed, people that cannot experience pain are prone to self-injury and develop persistent infections leading to a lower life expectancy (Axelrod & Hilz, 2003). However, when pain persists in the absence of a real or potential noxious stimulus, this mechanism will stop having a protective effect, becoming a tremendous health problem, known as chronic pain (Basbaum et al., 2009; Dubin & Patapoutian, 2010). Clinically, chronic pain is diagnosed when pain persists for longer than normal healing (varying from more than 3-6 months) (Shurtleff, 2018). At present, chronic pain has a burden impact in our society. It is one of the most prevalent health problems, affecting 20 % of the population (Dueñas et al., 2015; Zelaya et al., 2020).

Chronic pain and its impact in society

Chronic pain has been considered an extremely debilitating disease that dramatically reduces the quality of life for the people affected. Around 50 % of patients suffering chronic pain have reported that it limited their daily life activities, such as bending or walking up stairs. For 47 % of them, it affected their family life, and nearly 70 % of the patients suffered psychological distress or anxiety due to their chronic pain (Dueñas et al., 2015). Indeed, it has been associated to severe psychological disorders such as depression and suicide attempts. Pain also considerably affected sleep in more than 40 % of them (Dueñas et al., 2015; Karaman et al., 2014).

Due to all these health problems, chronic pain also has a strong economic impact in our society. It has been related to a drastic reduction in the effectiveness and productivity at work, increased absenteeism, and early retirement (Gaskin & Richard, 2012). In fact, it is the first cause of disability in

Spain, supposing 53% of the leading causes of total incapacity for work (Cavero Romero, 1996). Furthermore, pain is the cause of half of the visits to the emergency departments, consuming an important quantity of health services (Sanitat, 2015). Data from the United States shows that chronic pain supposes an estimated cost between 560 and 650 billion dollars only in this country (Gaskin & Richard, 2012).

With the current and predicted high increase in socioeconomic impact on the population, searching for more effective drugs for the prevention and treatment of chronic pain constitutes a significant challenge in biomedicine research. This has been partially complicated due to the wide diversity of chronic pain conditions that exist. To simplify pain study, they have been classified into different groups that share a common cause.

Types of chronic pain

According to their cause, we can distinguish between three major types of chronic pain: nociceptive, nociplastic, and neuropathic pain.

- **Nociceptive pain**: pain produced as a consequence of damaging non-neuronal tissue (Nijs et al., 2021). It is described as the body's reaction to potential or real harmful stimuli, and it is commonly produced by trauma, surgery, or chemical burns (Armstrong & Herr, 2022).
- **Nociplastic pain**: driven by altered nociception with no precise underlying mechanisms. It manifests without actual or threatened tissue damage or in the absence of any disease or lesion in the somatosensory pain system (Fitzcharles et al., 2021; IASP, 2017). Examples of nociplastic pain include fibromyalgia, irritable bowel syndrome, orofacial pain, etc. (Fitzcharles et al., 2021)
- **Neuropathic pain**: arising from a direct damage or disfunction in the pain neuronal system. It can be originated from trauma, infections, different diseases (e.g., diabetes, multiple sclerosis) and exposure to neurotoxic compounds such as chemotherapeutic drugs (Colloca et al., 2017).

Studying the molecular mechanisms underlying each of these chronic pain conditions could be the key to improving the quality of life for millions of people. However, before addressing them, we need to understand how pain can be produced.

Pain pathways

Pain is a complex phenomenon that integrates sensory, cognitive, and emotional components. It starts as an organism protective response from stimuli that can produce damage to the tissues, also known as noxious stimuli. The neural process by which noxious stimuli are detected and transmitted to the brain is termed nociception (Kendroud et al., 2022). Nociception comprises the activation of multiple physiological components and can be divided into 4 steps: transduction, modulation, transmission, and perception (Figure 1).

When a noxious stimulus strikes our body, it is detected by specialized primary afferent neurons called nociceptors (Tracey, 2012). If the stimulus reaches a determined threshold, the nociceptors will **transduce** it into an electrical signal: the action potential. Nociceptors are pseudounipolar neurons that innervate the skin and have their cell bodies (somas) located in the dorsal root ganglion (DRG) or trigeminal ganglion (TG). Thus, when a nociceptor detects a high threshold signal, it is converted into an action potential and **transmitted** through the axon from the site injured to the dorsal horn of the spinal cord, or, in the case of trigeminal neurons, to the nucleus of the cranial nerves. This signal is also exposed to **modulation** and can be amplified or reduced by different endogenous or exogenous mechanisms (Cross, 1994).

The dorsal horn is divided into different laminae and depending on the neuronal type and location, nociceptors end in different parts. Aδ nociceptors arrive to laminae I and to the deeper laminae V, meanwhile C nociceptors arrive to laminae I and II that are located more superficially (Basbaum et al., 2009) (Figure 2). In each of these parts, nociceptors release different neurotransmitters that would activate second-order neurons. These cells will send the information encoded in action potentials to the thalamus (spinothalamic tract neurons). From that point, the thalamus projects to forebrain regions transmitting the signal to the cerebral cortex (where pain is **perceived**). Pain perception would then occur in the brain when these action potentials are translated into a painful sensation.

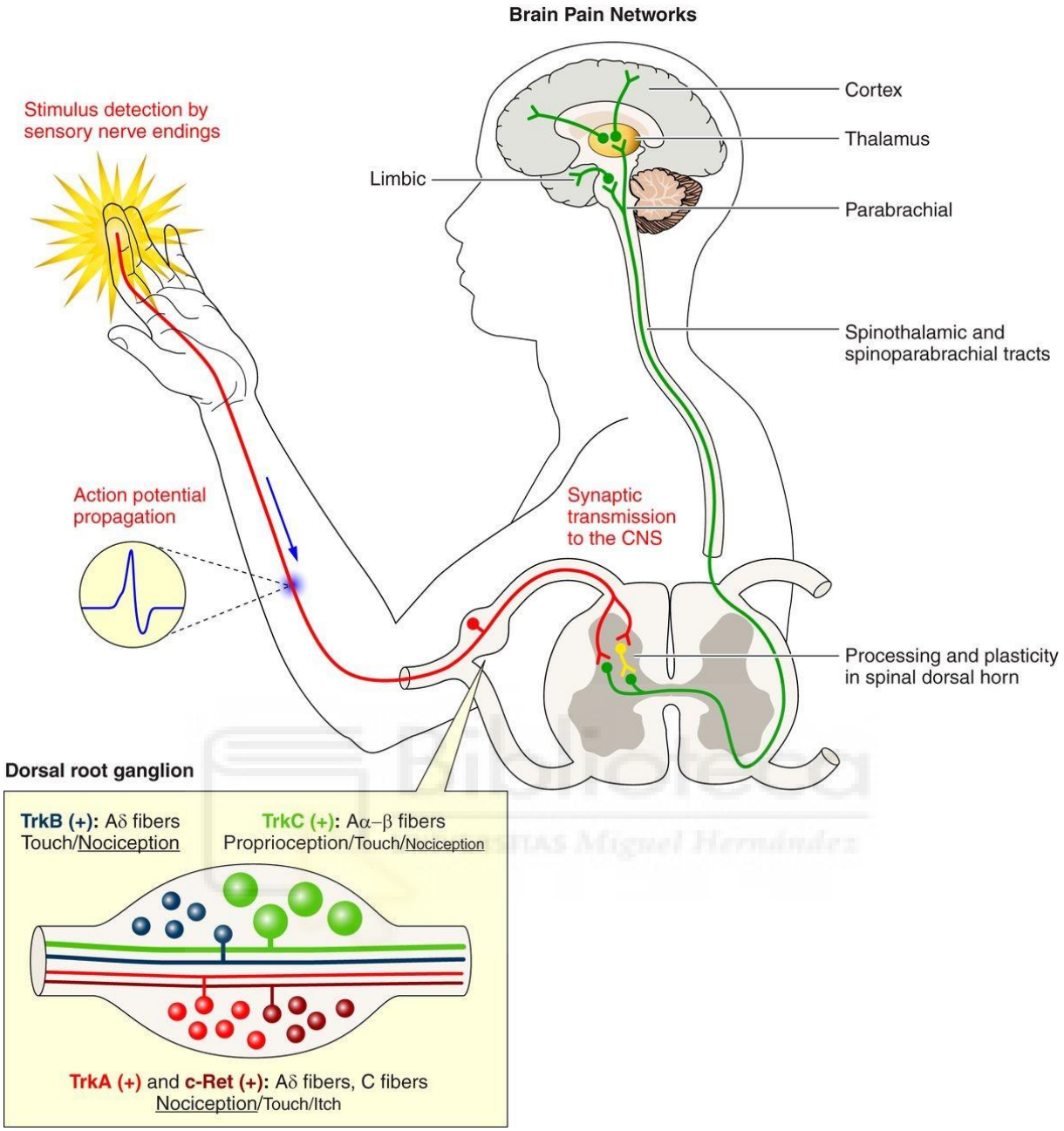


Figure 1. Schematic representation of pain pathways and processing. A noxious stimulus that can damage the body is detected by nociceptors (red). Nociceptors would transduce the stimulus into an action potential that is conducted through the axon to the dorsal horn of the spinal cord. There, the signal is transmitted with synapsis from nociceptors to interneurons (yellow) or second-order neurons (green). These second-order neurons project to the thalamus where they transmit the signal to the cerebral cortex. In this region, the electrical signal is converted into a painful sensation. Figure extracted from the review of Bourinet et al. (2014).

Anatomy and physiology of nociceptors

Primary afferent sensory neurons can be classified according to the diameter of the fiber in Aβ, Aδ, and C fibers. Aβ and Aδ fibers are myelinated and therefore, have faster conduction velocities of the electrical signal. Morphologically, they can be distinguished by measuring the soma diameter. Aβ fibers are large-diameter fibers that have been associated with non-nociceptive signals, such as light touch (Basbaum et al., 2009). Therefore, most of the pain studies have been focused on investigating the nociceptive fibers comprised by medium-diameter Aδ and the small diameter C-fibers. Due to the higher conduction velocity (5-30 $m \cdot s^{-1}$), Aδ fibers have been considered as the acute and initial detectors of pain (Djouhri & Lawson, 2004). Meanwhile, C-fibers, that lack a myelin sheet and have conduction velocities between 0.4-1.4 $m \cdot s^{-1}$, have been linked to the slow and diffused pain (Dubin & Patapoutian, 2010). These characteristics have driven to the association of C-fibers with persistent pain.

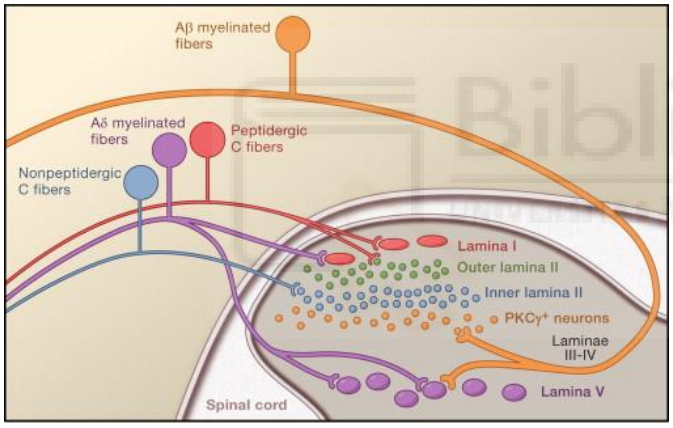


Figure 2. Different fiber types found in the DRG neurons and their connections with dorsal horn. Image showing each fiber type connecting with different laminae of the dorsal horn of the spinal cord. Figure extracted from the review of Basbaum et al. (2009).

C nociceptors form a very heterogeneous group comprising different neuronal types (Zheng et al., 2019). Classically, they have been classified into two major classes: nonpeptidergic and peptidergic C nociceptors (Basbaum et al., 2009). Peptidergic neurons (IB4(-)) are characterized by the expression and release of neuropeptides such as substance P and the calcitonin-gene related peptide (CGRP) (Basbaum et al., 2009). These neurons also express the TrkA neurotrophin receptor that detects the nerve growth factor (NGF). Nonpeptidergic neurons (mainly IB4(+)) express the c-Ret neurotrophin receptor that detects the glial-derived neurotrophic factor (GDNF). They also express G-protein coupled receptors (from Mrg family) and P2X3 receptors (Basbaum et al., 2009). These populations can also be differentially labelled with the IB4 isolectin because it primarily binds to the non-peptidergic neurons (named as IB4(+) neurons) (Villalba-Riquelme et al., 2022). Thus, with the aim of

elucidating the mechanisms underlying the pain symptoms, we designed this thesis to investigate the alterations produced on C nociceptors separating between IB4(-) and IB4(+) nociceptors.

Ion channels in pain

The molecular mechanisms by which nociceptors can transduce noxious stimuli into an electrical signal can be explained with the ion channels. Ion channels are transmembrane proteins that can allow the movement of selective ions across the cell membrane, resulting in an electrical signal (Grider et al., 2022; Huettner, 2013). In nociceptors, a noxious stimulus and/or the products derived of the reaction of the body to it, can activate sodium (Na^+) and calcium (Ca^{+2}) permeable ion channels, such as the transient receptor potential (TRP) channels. This will allow an initial influx of cations inside the cell that would increase the membrane potential depolarizing the cell.

If this response arrives to the activation threshold of voltage-gated sodium (Na_V) channels, they will allow the pass of Na^+ ions producing a bigger entrance of cations to the cell. This evokes a stronger and fast depolarization of the cell membrane. These channels inactivate fast stopping the Na^+ entry when the action potential arrives to the depolarization peak. At this time, the voltage-gated potassium channels (K_V) start opening, allowing the outflux of potassium ions (K^+) from the inside of the cell. As a result, the cell repolarizes to the resting membrane potential (RMP). Due to the slower closing kinetics of the K_V, the K^+ ions continue leaving the cell until they close, resulting in a reduction of the membrane potential below the RMP, process termed as the cell hyperpolarization (Grider et al., 2022). Finally, the Na^+-K^+ ATPase would reestablish the ionic balance returning the voltage to the resting membrane potential (RMP) values by moving Na^+ ions out of the cell and introducing K^+ ions (Grider et al., 2022).

Role of TRP channels as pain sensors

TRP channels form a key superfamily of ion channels involved in pain transduction. Due to the crucial role in sensory physiology, these channels can be found in a great variety of organisms from invertebrates to humans (Venkatachalam & Montell, 2007). The structure of TRP channels is characterized by 6 transmembrane helices that allow the flow of cations between the outside and inside compartments of the cell (Venkatachalam & Montell, 2007). As explained in the previous part, upon detection of noxious stimulus, these channels allow the influx of Na^+ and Ca^{+2} ions, depolarizing the cell and, when arriving to the Na_V activation threshold, triggering the generation of an action potential. The ionic selectivity and activation mechanisms of these channels have a strong variation between the different subtypes (Venkatachalam & Montell, 2007).

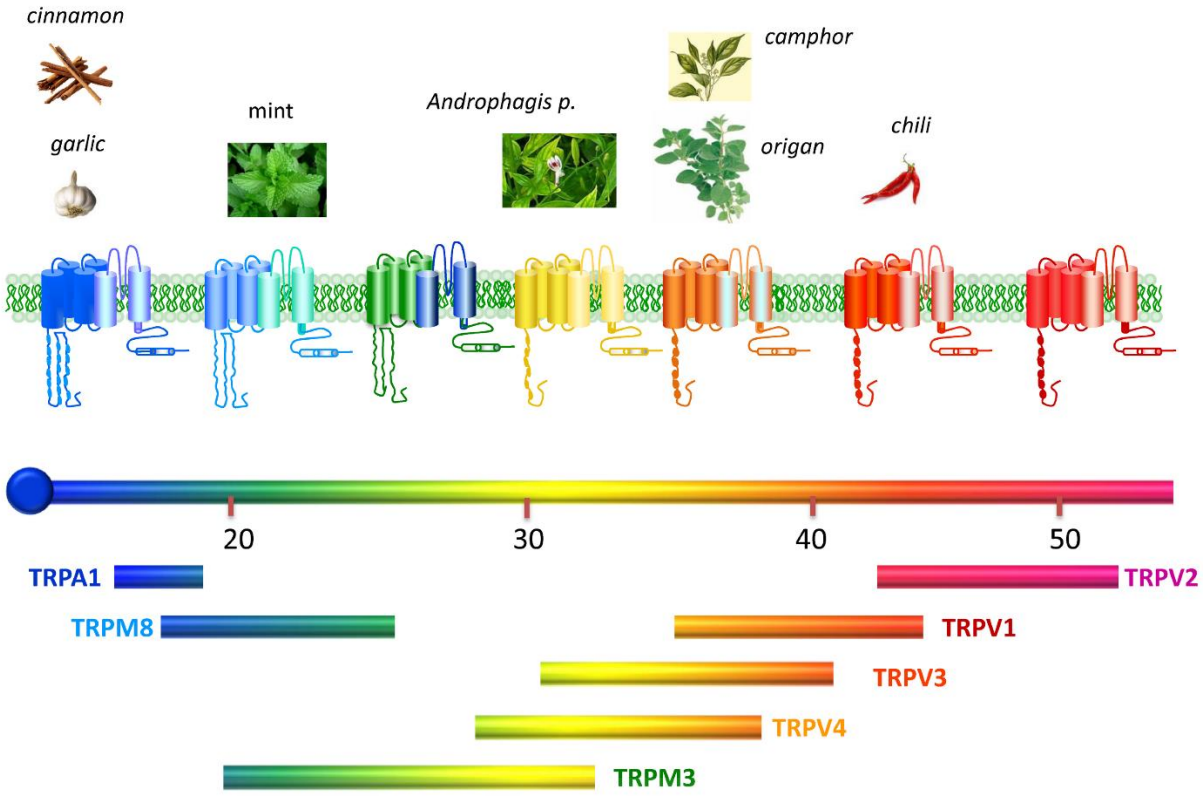


Figure 3. Different TRP channels working as temperature sensors and pain transducers in the mammalian DRG neurons. At present, 7 distinct TRPs have been discovered to act as thermal sensors (also known as thermoTRPs): TRPA1, TRPM8, TRPM3, TRPV4, TRPV3, TRPV1 and TRPV2. These channels display different thermal sensitivities that vary from detecting noxious cold to noxious hot. The temperature activation range for each channel is: ≤ 17 °C for TRPA1, ≤ 25 °C for TRPM8, ≥ 30 °C for TRPM3, ≥ 34 °C for TRPV4, ≥ 36 °C for TRPV3, ≥ 43 °C for TRPV1, and ≥ 52 °C for TRPV2. These channels can also be activated by the specific natural and/or chemical synthesized compounds indicated above. Figure extracted from (Ferrandiz-Huertas et al., 2014).

To date, more than 50 different subtypes of TRP channels have been found. According to their characteristics, these channels can also be grouped in 7 subfamilies: TRP Vanilloid (TRPV), TRP Canonical (TRPC), TRP Melastatin (TRPM), TRP NOMPC (TRPN), TRP Ankyrin (TRPA), TRP Polycystic (TRPP), and TRP Melastatin-Like (TRPML) (Montell, 2005; Venkatachalam & Montell, 2007). In mammals, members of the families TRPV, TRPM and TRPA are expressed on the cell membrane of the DRG and TG neurons (Venkatachalam & Montell, 2007). Among them, TRPV1, TRPA1 and TRPM8 have been postulated as the main TRP channels involved in pain pathways, although other thermoTRP channels such as TRPM3, TRPV2, TRPV3, and TRPV4 are also starting to be associated with pain sensation (Cabañero et al., 2022; Fischer et al., 2010; Vandewauw et al., 2018).

TRPV1

TRPV1 was the first pain transducer detected. It can be mainly found in the medium and small-diameter primary sensory neurons of the DRG, TG and nodose ganglia (Vay et al., 2012). However, it has also been localized in the brain and non-neuronal tissues (De Toni et al., 2016; Menigoz & Boudes, 2011).

TRPV1 is a polymodal ion channel that can be activated by a wide variety of stimuli known to cause pain *in vivo*, such as noxious heat (Tª ≥ 43 °C, Figure 3), low pH (pH < 6), voltage, and several chemical agents like lipids, ethanol, and capsaicin (the pungent compound found in hot peppers) (Tracey, 2012). Upon activation, it allows the Na^{+} and Ca^{+2} pass through the membrane with a selectivity ratio (P_{Ca+2}:P_{Na+}) of 3.8 for heat, and 9.6 for vanilloids (Venkatachalam & Montell, 2007).

Alterations of TRPV1 function have been found in numerous pain disorders like migraine and chemotherapy-induced peripheral neuropathy (CIPN) (Villalba-Riquelme et al., 2022; Yakubova et al., 2021). It can also be modulated by the proinflammatory mediators released during tissue damage and inflammation, that can reduce its thermal and pH threshold having a proalgesic effect on inflammatory pain disorders (Cesare & McNaughton, 1996). As a result, TRPV1 has emerged as a promising therapeutic target for pain modulation. Indeed, TRPV1 modulators such as capsaicin patches have already been widely used in the clinical practice to minimize the pain symptoms and recent studies are designing novel TRPV1 antagonists with a safer pharmacological profile showing promising results as analgesic tools (Nikolaeva-Koleva et al., 2021; Privitera & Anand, 2021; Serafini et al., 2018).

TRPA1

TRPA1 is one of the most promiscuous TRP channels known. It can act as a chemical sensor, binding to more than 90 different chemical compounds, such as environmental and metabolic irritants (e.g., allyl isothiocyanate (AITC), acrolein, cinnamaldehyde, benzyl isothiocyanate, safranal) and by-products of oxidative stress and hydrogen peroxide (Bautista et al., 2006; Zygmunt & Högestätt, 2014). Its response to oxidative stress together with the direct or indirect responses to proinflammatory agents (e.g. bradykinin), has attributed an important role of TRPA1 for inflammatory pain (Bourinet et al., 2014). Indeed, and due to its wide distribution around the body, it has been linked to numerous inflammatory disorders. This includes asthma and chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, endometriosis, inflammatory bowel disease, atherosclerosis, inflammatory skin diseases (e.g, psoriasis), and neurodegenerative inflammatory diseases (e.g. Parkinson, Alzheimer Disease) (Landini et al., 2022).

Apart from its role as a chemical detector, TRPA1 is also known as a mechanical and thermal detector. It can be activated by noxious cold (Tª ≤ 17 °C) (Story et al., 2003) (Figure 3). Thus, it has been associated with the painful sensation evoked by low temperatures. However, there is still controversy according to its level of relevance in thermal or mechanical sensation. Opposite results have been obtained with mice lacking the channel. Bautista et al. (2006) showed that ablation of TRPA1 channel did not affect cold sensation in mice, whereas Kwan et al. (2006) observed diminished responses to lower temperatures than 0 °C. Despite these differences, its role on pain pathologies has been widely documented. As an example, mutations of the TRPA1 gene that result in a gain of function of the protein were responsible of the familial episodic pain syndrome (FEPS). This syndrome is characterized by debilitating pain that appears after fasting or physical stress (Kremeyer et al., 2010).

TRPA1 has been found in neuronal (primary sensory neurons from DRG, TG and nodose ganglia) and non-neuronal tissues (respiratory tract cells, enterochromaffin cells, keratinocytes, melanocytes, synoviocytes, gingival fibroblasts, epithelial cells, mast cells and β-pancreatic cells (Landini et al., 2022)). Its expression on glial cells, such as astrocytes, oligodendrocytes and Schwann cells can also contribute and modulate the nociceptive pain pathway (Landini et al., 2022).

TRPM8

The TRPM8 channel has been linked to cool sensation by numerous studies. First, it can be found in neurons that are sensitive to low temperatures from DRG and TG (Tª < 25 °C) (Figure 3). It can also be activated by chemicals that elicit a cooling sensation, like menthol, icilin, and eucalyptol (Peier et al., 2002). Indeed, TRPM8$^{-/-}$ mutant mice did not show sensitivity to cool temperatures (Bautista et al., 2007). Nevertheless, these mice still showed avoidance of cold surfaces (< 10 °C), although it was reduced, suggesting that TRPM8 is not the unique channel involved in sensing noxious cold (Bautista et al., 2007).

Alterations on the TRPM8 activity have been linked to different pain disorders such as migraine or CIPN (Alarcón-Alarcón et al., 2022; Chukyo et al., 2018; Liu et al., 2013; Villalba-Riquelme et al., 2022). Its crucial role on pain pathophysiology has driven to its interest as a potential analgesic target with several clinical trials testing modulators of the channel in pain conditions (Fallon et al., 2015; Kerckhove et al., 2019).

Role of other TRP channels in pain

Although less investigated, other TRP channels have also been found in sensory neurons and are also being studied in pain signaling for its detection of thermal and/or noxious stimulus. This is the case of TRPV2, TRPM3, TRPV4, and TRPV3. TRPV2 is expressed on Aδ fibers where it is activated at

Tª > 52 °C, transducing noxious heat (Tracey, 2012). TRPM3 is also activated by noxious heat and is expressed in C fibers in the DRG and trigeminal ganglion (Tracey, 2012). Meanwhile, TRPV3 and TRPV4 can sense warm temperatures between 25 °C and 35 °C (Figure 3).

All these channels have been linked to different pain disorders and future research would be needed to clarify their contribution to pain sensation. For instance, TRPV3 mutations have been linked to the presence of migraine in the population (Carreño et al., 2012). TRPV4 contributed to the pain symptoms in several models of the peripheral neuropathy evoked by chemotherapeutic agents (CIPN) (Alessandri-Haber et al., 2008; De Logu et al., 2020; Sánchez et al., 2020). TRPV2 has also been altered in a CIPN model, although its specific contribution to the related pain symptoms remains unknown (Hori et al., 2010). Finally, recent data has shown that TRPM3 participated in the heat hyperalgesia and neuropathic pain evoked after nerve injury (Su et al., 2021). Furthermore, its high expression in the trigeminal nerves has also placed this channel as a potential contributor to migraine (Cabañero et al., 2022).

Consequently, TRP channels have demonstrated a crucial role in pain pathophysiology as noxious stimuli transducers. But, in addition to them, the action potential is generated by the activation of Na_V and K_V ion channels in the neurons. Thus, for understanding the pain pathologies we should investigate these channels.

Role of Na_V channels in pain

Mammalian DRG neurons express 5 different Na_V channels: $Na_V1.1$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$. $Na_V1.1$ and $Na_V1.6$ are predominantly expressed in large DRG neurons (Goldin et al., 2000; Ho & O'Leary, 2011). Meanwhile, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are the predominant Na_V subtypes expressed in nociceptors (Ho & O'Leary, 2011). There, $Na_V1.7$ has been proposed to contribute to the threshold and upstroke phase of the action potential (Meents et al., 2019). $Na_V1.8$ seems to produce most of the inward current responsible for the action potential depolarization (rising phase) and to be responsible of the repetitive firing evoked under stimulation (Ho & O'Leary, 2011; Lai et al., 2003). Meanwhile, $Na_V1.9$ is a slowly-gating ion channel that mediates a persistent sodium current (Tracey, 2012). Due to its low activation voltage, it has been linked to set the resting membrane potential and the responses to subthreshold stimulus (Tracey, 2012). In a smaller extent, $Na_V1.6$ can also be found in nociceptors, where it can contribute to the conduction of the action potential (Black et al., 2002). The specific contribution of each Na_V subtype to the action potential is indicated in Figure 4.

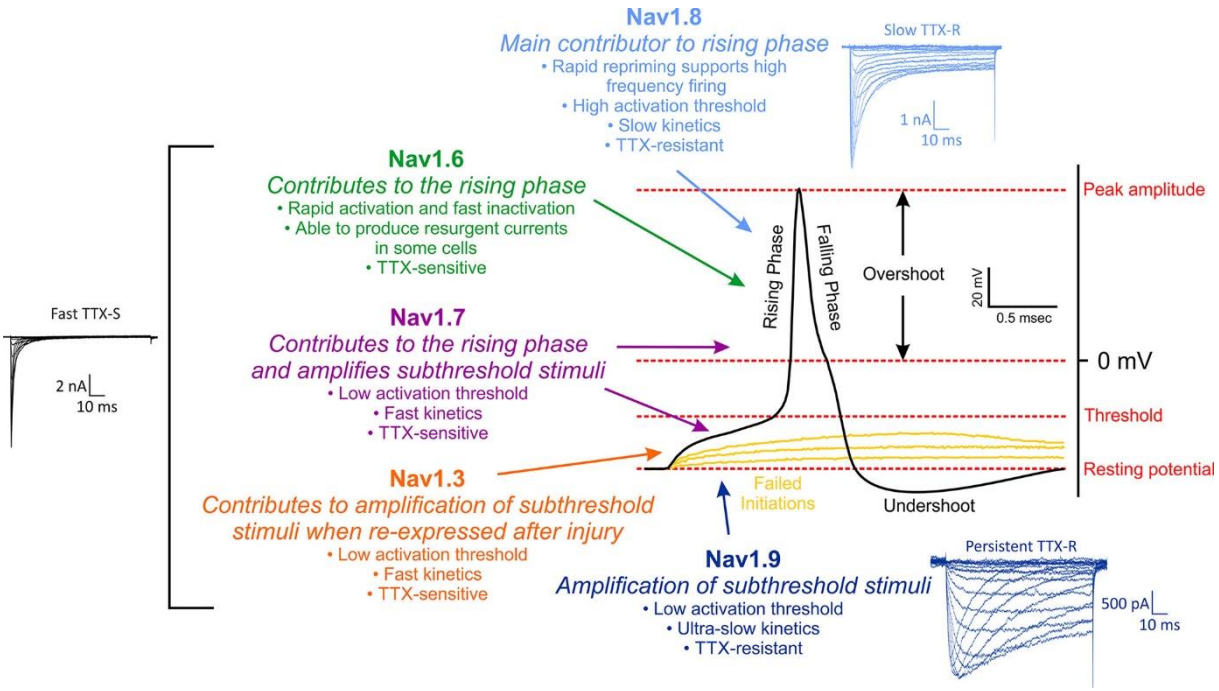


Figure 4. Contribution to the action potential characteristics of the major Na_V channels linked to pain in nociceptors. Due to their low activation threshold, subthreshold stimuli can be amplified by $Na_V1.3$, $Na_V1.7$, and $Na_V1.9$ ion channels. Then, the rising phase of the action potential is mainly produced by $Na_V1.8$ activity, although $Na_V1.7$ and $Na_V1.6$ have also been shown to participate in this depolarization phase. These channels can also be classified according to their sensitivity to tetrodotoxin (TTX) as TTX sensitive or TTX resistant. Figure extracted from the review of Bennett et al. (2019).

The discovery of the congenital indifference to pain generated in patients with loss of function mutations of $Na_V1.7$ pointed towards its high relevance in pain sensation (Cox et al., 2006). Indeed, mutations in the $Na_V1.7$ have also been determined as being the cause of different peripheral pain disorders such as erythromelalgia (Dib-Hajj et al., 2005). The crucial contribution of $Na_V1.7$ to pathological pain states is also shared with the $Na_V1.8$ channel. Numerous $Na_V1.8$ genetic gain-of-function mutations have been found in patients with painful peripheral neuropathies and blockage of $Na_V1.8$ activity attenuated pain behaviors (Faber et al., 2012; Jarvis et al., 2007). Interestingly, a different Na_V channel, called $Na_V1.3$, that was undetectable in the adulthood, was reexpressed in injured sensory neurons (Waxman et al., 1994). Thus, expression of $Na_V1.3$ has also been hypothesized to contribute to exacerbate the pain symptoms produced in peripheral neuropathies.

Role of K_V channels in pain

Elucidating the physiological role of K_V channels has been challenged by the huge quantity of subtypes that are expressed. Only in nociceptors it is possible to find predominantly 11 different subtypes: K_V1.4, K_V2.1, K_V2.2, K_V3.4, K_V4.1, K_V4.3, K_V7.2, K_V7.3, K_V7.5, and the silent K_V9.1 and K_V9.3 (Du & Gamper, 2013). These channels have substantial differences based on their inactivation kinetics and, classically, they have been grouped according to their current properties into two groups. The K_V channels producing a fast-inactivating current (A-type, I_{KA}), mainly represented by K_V1.4, K_V3.4, K_V4.1, and K_V4.3 in nociceptors (Zemel et al., 2018), and the channels responsible for the delayed-rectifying slowly inactivating (I_{KDR}) current, K_V2.1, K_V2.2, K_V7.2, K_V7.3, and K_V7.5 (Luo et al., 2011; Zheng et al., 2019).

To date, poor information has been provided on the specific role of these K_V subunits to pain disorders. The absence of specific pharmacological blockers constituted an obstacle for elucidating their individual contribution to DRG electrogenicity (Zemel et al., 2018). However, a general role for these channels in regulating cell excitability has been demonstrated. Specifically, K_V1.4 could regulate AP duration and firing frequency by allowing the entry of K^+ ions at low voltages ($V_{1/2}$ ~ -14 mV) (Park et al., 2020). K_V3.4, that shows a high activation threshold ($V_{1/2}$ ~ 20 mV), has been linked to shape the initial AP repolarization (Vydyanathan et al., 2005). The fast recovery from inactivation and the low activation threshold of K_V4.3 (-50 to -60 mV), suggests that this channel could be implicated in the repolarization of the action potential (Du & Gamper, 2013). K_V2.1 and K_V2.2 would also contribute to the repolarization and hyperpolarization phases, since inhibition of these channels depolarized the membrane during interspike interval, promoting repetitive firing in superior cervical ganglion neurons (Liu & Bean, 2014). K_V7.2, 7.3, and 7.5, responsible for the M-current, have been attributed to set the resting membrane potential and regulate the action potential firing threshold (Barkai et al., 2017).

The relevance of these channels on nociception has been supported by the lower K_V activities found in several pain pathologies. Between these channels, K_V1.4 was the first channel identified in nociceptors and the most widely described to be affected in pain pathologies (Zemel et al., 2018). In painful diabetic neuropathy, lower I_{KA} and K_V1.4 mRNA was reported by Cao et al. (2010). Similar effect was observed under temporomandibular joint inflammation, where K_V1.4 immunoreactivity was decreased and related to the generation of allodynia (Takeda et al., 2008). Besides, downregulation of K_V1.4, 2.2, and 4.2 mRNAs was detected after transection and chronic constriction injury of the sciatic nerve (Kim et al., 2002; Park et al., 2003). Reduced K_V1.1 mRNA expression was also found in the neuropathic pain condition induced by oxaliplatin treatment (Descoeur et al., 2011).

In support of this tenet, the anti-nociceptive effects shown by the agonists of these channels has been widely described. Nodera et al. (2011) found that the K_V7 activator, retigabine, protected against the painful nerve hyperexcitability caused by cisplatin in mice. This is also the case of diclofenac, a compound that enhances I_{KA} currents and was able to reverse the pain symptoms in a bone cancer model (Duan et al., 2012).

Other ion channels implicated in pain

Other ion channels that have shown a key role on nociceptors excitability and pain control include the voltage-gated calcium channels (Ca_V), "two-pore domain" potassium channels (K_2P), hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, acid sensing ion channels (ASICs), Piezo, and the purinergic P2X receptors (Benarroch, 2015; Busserolles et al., 2020). A brief summary of the major characteristics and the contribution of these ion channels to pain is collected in Table 1.

The expression of any of these ion channels can change under pathological conditions, leading to chronic pain. However, as mentioned before, a wide variety of pain disorders can be found in society. Regarding the type of chronic pain, different alterations on the pain pathways occur. Therefore, to better understand the molecular changes implicated in a determined pain condition, we need to focus on the specific pathology.

For neuropathic pain, one of the disorders with a drastic and increasing impact in the human population is the peripheral neuropathy evoked by chemotherapeutic drugs (CIPN). Due to its clinical relevance, in this thesis we investigated the functional and molecular mechanisms underlying the pain symptoms associated to CIPN.

Table 1. Summary of the main characteristics and their implication in pain of Ca_V, K_2P, HCN, ASICs, Piezo and P2X ion channels expressed in the peripheral nervous system.

Channels	Characteristics	Pain implication	Reference
Voltage-gated calcium channels (VGCC)			
$Ca_V1.2$ $Ca_V2.2$ $Ca_V3.2$ $Ca_V3.3$	Modulation of neuronal excitation and peripheral release of neuropeptides and glutamate.	Upregulated in experimental models of inflammatory and neuropathic pain.	(Tomita et al., 2019; Watanabe et al., 2015)
Two-pore domain potassium K2P channels			
THIK-2 TASK1 TWIK1 TREK1 TASK2 TRESK TRAAK TREK2 TWIK2	Characterized by being constitutively opened. Responsible of the K^+ background current that controls resting membrane potential at subthreshold voltage.	TRESK mutation associated with migraine. Decreased mRNA expression of TASK2 four days after inflammation induced by CFA. TREK1 and TRAAK reduced mRNA expression in DRG neurons of a mice model of oxaliplatin-induced peripheral neuropathy.	(Lafrenière et al., 2010) (Marsh et al., 2012) (Descoeur et al., 2011)
Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels			
HCN1 HCN2	Activated by hyperpolarizing potentials, control the hyperpolarization phase of the action potential.	Inhibitors of HCN channels showed antinociceptive effects. Upregulation of HCN channels in the sciatic nerve CCI model and chemotherapy-induced pain models.	(Luo et al., 2007) (Jiang et al., 2008) (Liu et al., 2018; Resta et al., 2018)
Acid sensing ion channels			
ASIC1a, ASIC1b ASIC2a, ASIC2b ASIC3	Activated by extracellular H^+. These channels act as chemo-electrical transducers in nociceptors.	Implicated in the pain symptoms generated by the tissue acidosis produced in models of inflammatory pain.	(Walder et al., 2010)
Piezo			
Piezo1 Piezo2	Mechanosensitive ion channels involved in mechanotransduction.	Associated with migraine pain.	(Della Pietra et al., 2020)
Purinergic P2X receptors			
P2X3	Activated by ATP, involved in the nociceptive response to ATP.	Implicated in the pain response to damaged and inflamed tissues.	(Oliveira et al., 2009)

Chemotherapy-Induced Peripheral Neuropathy (CIPN)

The discovery of chemotherapy drugs has improved enormously the survival of many different cancer types (André et al., 2004; Davidson, 1996; Ozols, 1992). However, their clinical use is limited due in part to the development of a chemotherapy-induced peripheral neuropathy (CIPN) consequent to the treatment. CIPN is estimated to affect between 19-85% of the patients, with a prevalence of 68.1% within the first month after chemotherapy, 60% after 3 months, and 30% after 6 months or more (Seretny et al., 2014). CIPN symptoms include paresthesia and dysesthesia with spontaneous pain, thermal, and mechanical hyperalgesia and/or allodynia (Zajączkowska et al., 2019). These symptoms predominantly arise in the feet and hands, commonly known as a "glove and stocking" distribution (Burgess et al., 2021). As a consequence, patients developed numerous limitations in their daily activities, such as manipulating small objects, showing a drastic reduction in their quality of life (Prutianu et al., 2022). These symptoms are so severe that they can force the reduction or discontinuation of the treatment in up to 30 % of the patients, compromising the patients' survival (Prutianu et al., 2022).

Despite its high prevalence and impact, there is no effective treatment approved for CIPN symptoms. At present, only duloxetine has been recommended in the guidelines of the American Society of Clinical Oncology (ASCO) (Loprinzi et al., 2020). However, it showed a moderate effect in reducing CIPN symptoms of chemotherapy-treated patients (Loprinzi et al., 2020; Smith et al., 2013).

The poor CIPN management has been mainly attributed to the lack of understanding of the molecular mechanisms underlying the peripheral neuropathy. Investigation of these mechanisms and its sexual dimorphism would be crucial for elucidating potential molecular targets that could revert the pain symptoms and improve the patients' quality of life.

Pathobiology of CIPN

Chemotherapy drugs exert its major effect in the peripheral nervous system (PNS) due to its physiology. Peripheral neurons are characterized by presenting long axons. Cells with longer axons might be more affected due to their higher metabolic requirements (Gutiérrez-Gutiérrez et al., 2010; Han & Smith, 2013). The central nervous system is highly protected due to the blood-brain barrier (Hu et al., 2019). However, the PNS has a dense vascularization with high permeability capillaries that allow the entry of low and high molecular weight substances. As a consequence, chemotherapeutic drugs can preferentially accumulate within the DRG injuring these cells (Jimenez-Andrade et al., 2008).

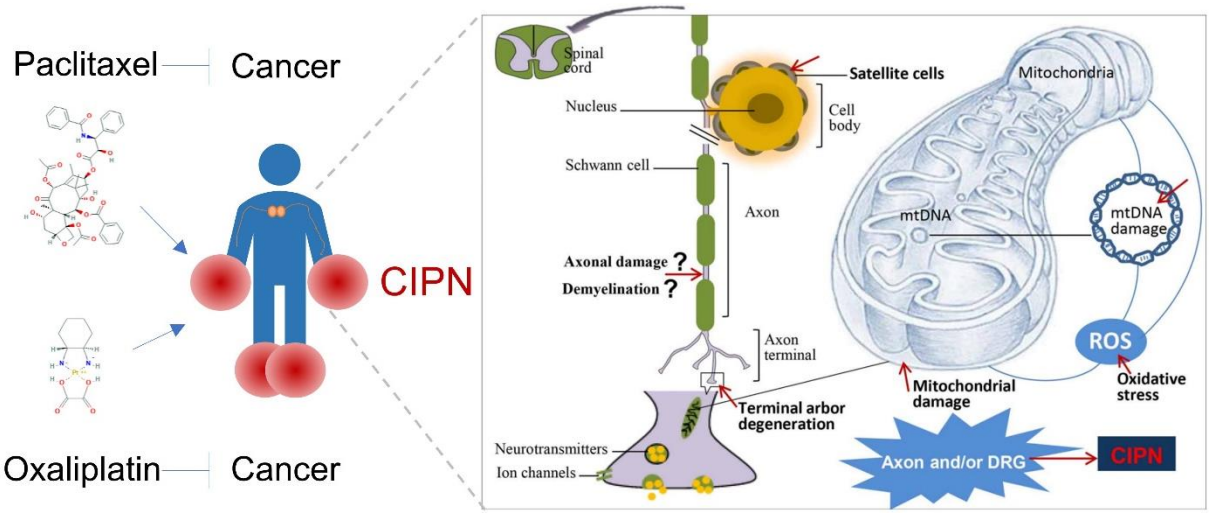


Figure 5. Pathophysiology of CIPN. Chemotherapeutic drugs used for cancer treatment produce a peripheral neuropathy (CIPN) that can be translated into acute and chronic pain. These painful symptoms primarily arise with a glove-an-stock distribution (left). At a physiological level, the neuropathy can be explained due to the damaging effects that exert these chemotherapeutic agents on different components of the peripheral nervous system (right). Figure modified from the review of Han and Smith (2013).

At the cellular level, chemotherapy effects result in complex structural and molecular alterations of the sensory neurons including loss of intra-epidermal nerve fiber (IENF), mitochondrial damage, neuroinflammation, DNA damage, myelin damage, immunological processes, ion channel alterations, and oxidative stress (Han & Smith, 2013; Zajączkowska et al., 2019).

IENF is one of the most prominent structural changes developed by patients and used by clinicians to diagnose peripheral neuropathies (Mangus et al., 2020). Following chemotherapeutic treatment, the DRG neurons underlie terminal arbor degeneration, which results in a reduction of IENF density that has been previously linked to the severity of the painful symptoms (Han & Smith, 2013).

Another structural change observed was the degeneration of myelin sheath with reduction in axonal diameter and segmentation of myelin (Chine et al., 2019). Mitochondrial disfunction impairs the generation of neuronal energy and increases ROS production (Colvin, 2019). Furthermore, non-neuronal mechanisms have also been found to condition CIPN. Induction of immune cells and

alterations in inflammatory cytokines usually occur after treatment, sensitizing DRG neurons (Colvin, 2019).

All these changes result in alterations on the ion channel activity responsible for the sensitized neuronal activity. Furthermore, several chemotherapeutic drugs have shown a direct effect on the ion channels activity and/or through modulation of their signaling pathways (Li et al., 2015; Rimola et al., 2021).

The specific symptoms and mechanisms underlying CIPN vary depending on the type of treatment. There are six main groups of chemotherapeutic drugs: taxanes, platinum-based antineoplastics, vinca alkaloids, epothilones, proteasome inhibitors, and immunomodulatory drugs (Zajączkowska et al., 2019). Among them, platinum-based and taxanes are some of the most prescribed and most toxic agents (Zajączkowska et al., 2019). Therefore, in the present thesis, we aimed to elucidate molecular and functional changes underlying the chronic pain elicited by the most common taxane-like agent, paclitaxel, and the platinum-based agent, oxaliplatin.

Paclitaxel

Paclitaxel (Taxol®) is one of the most effective drugs used for the treatment of solid tumors. It is the first-line treatment of breast and ovarian cancer, and it has also shown efficacy against non-small lung cell cancer and Kaposi's sarcoma (Abu Samaan et al., 2019; Markman, 1991; Ramalingam & Belani, 2004). However, between 60-90% of patients receiving this therapy develop a paclitaxel-induced peripheral neuropathy (PIPN) that can be eventually translated into acute and chronic pain (Colvin, 2019).

PIPN is one of the major side-effects following paclitaxel treatment. It is characterized by sensations of numbness, tingling, burning, shooting, and shock-like pain and usually involve the hands and feet (Loprinzi et al., 2011). The severity of these symptoms can limit its clinical use impacting on patients' survival (da Costa et al., 2020; Seretny et al., 2014). Furthermore, 97 % of paclitaxel patients would develop painful CIPN and it would persist in 30 % of the patients after cessation of the treatment (da Costa et al., 2020; Seretny et al., 2014). Nevertheless, the mechanisms underlying the pain symptoms remain to be elucidated.

Molecular mechanism of PIPN

Paclitaxel has an anti-tumor effect because it can bind to beta-tubulin and stabilize the microtubules polymerization. As a consequence, cells cannot divide leading to apoptosis (Xiao et al., 2006). This therapeutic effect against cancer cells, become neurotoxic on DRG neurons. Microtubules

are essential for the correct axonal transport of proteins from the cell soma to the terminals. Binding of paclitaxel to tubulin interferes with normal microtubule dynamics, resulting in overpolimerization and enhanced microtubule bundling in axons (Albertini et al., 1984).

This effect, together with the inflammatory signaling evoked, the altered Ca^{+2} homeostasis, and mitotoxicity have been hypothesized to drive the axonal degeneration produced following paclitaxel treatment (Crish et al., 2019). As a result, the mechanical and electrical properties of DRG neurons are altered (Eldridge et al., 2020). The current hypothesis of the pathophysiology of PIPN has been summarized on Figure 6.

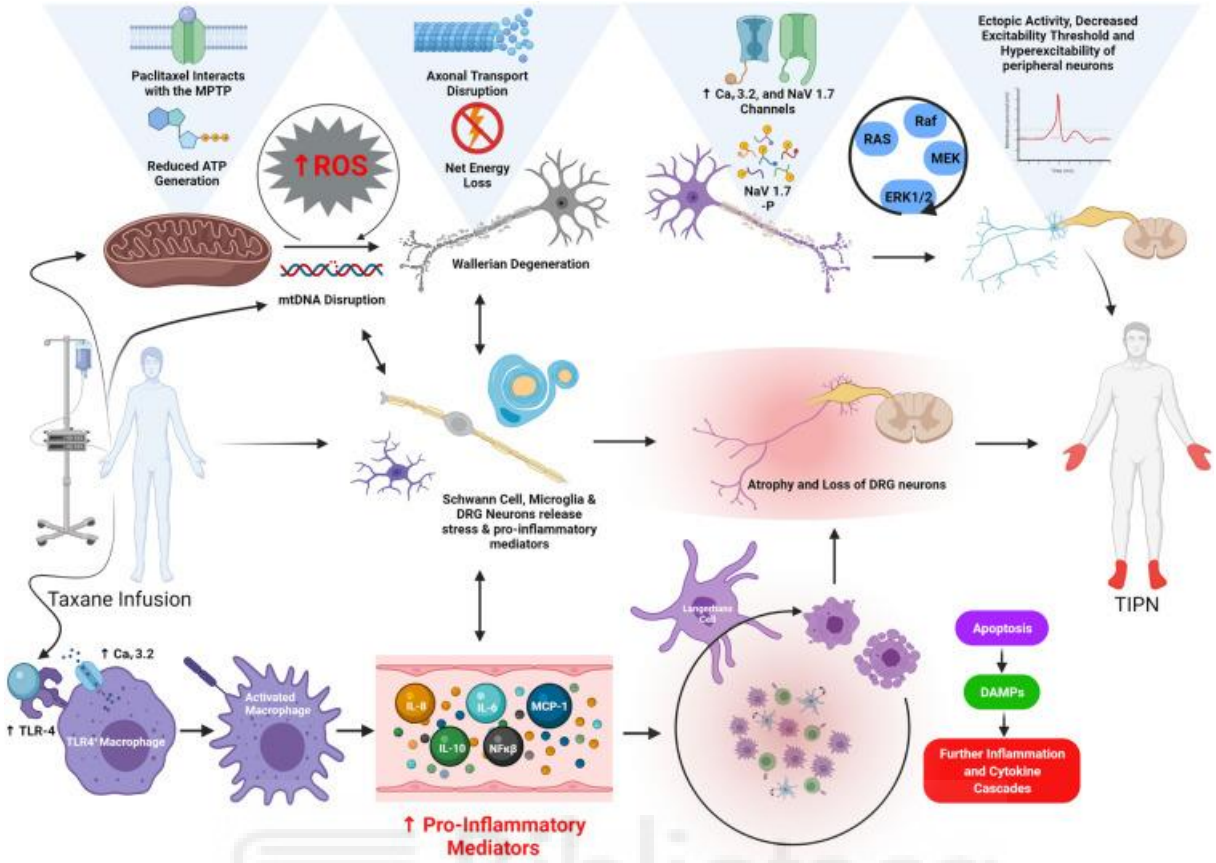


Figure 6. Current hypotheses of the pathophysiology of PIPN. Paclitaxel infusion drives to pro-inflammatory cascades through interaction with TLR4 and macrophages. This process induces the release of IL-6, IL-8, IL-10, and MCP-1 by Langerhans cells. Inflammatory signaling and oxidative stress markers are also increased in Schwann cells, microglia and DRG neurons. The oxidative stress generated interferes with mitochondria, causing energy loss and intracellular damage (DAMPs). Paclitaxel also binds to tubulin, interfering with normal microtubule dynamics and axonal transport. As a result, the activity of different ion channels such as Na_V, Ca_V, K_V, and TRP is altered causing the neuronal hyperexcitability. DAMPs: damage-associated molecular patterns. MCP-1: monocyte-chemoattractant protein-1. MPTP: mitochondrial permeability transition pore. Figure extracted from the review of Burgess et al. (2021).

All these changes are translated into the altered functionality of the DRG neurons, driving the neuropathic pain symptoms. Indeed, previous studies on treated animals showed that paclitaxel could increase the electrical activity on the sensory neurons (Zhang & Dougherty, 2014). Hence, understanding the alterations on the neuronal ion channels that are responsible of the increased excitability would be essential for reverting the symptoms. Alterations on DRG neurons electrical

activity can be produced mainly by alterations in the Na^+, K^+ and Ca^{+2} ion channels (Waxman & Zamponi, 2014).

Na_V channels have been considered as potential drivers of the spontaneous activity generated after paclitaxel. From the Na_V subunits, Nav1.7 has been the most studied and related to paclitaxel produced excitability. Y. Li et al. (2018) showed that $Na_V1.7$ was upregulated after paclitaxel treatment in rats and application of ProTx II reduced behavioral signs of CIPN. In a recent article, increased $Na_V1.7$ vesicular trafficking and surface expression was observed, however this effect decreased with higher concentrations of paclitaxel (Akin et al., 2021).

Despite the essential role of K_V channels controlling the generation of action potentials, there is poor literature analyzing them. The huge diversity of K_V channels difficulties its isolation. Altered mRNA expression with increased levels of $K_V1.2$, $K_V11.3$ and HCN1 and reduced $K_{ir}1.1$, $K_{2P}1.1$ and $K_{ir}3.4$ were reported (Zhang & Dougherty, 2014). There are also initial results indicating that K_V could be a good target for treating CIPN since the $K_V7.2/7.3$ activator retigabine reduced paclitaxel induced hyperexcitability (Li et al., 2019). However, additional studies are needed to confirm its implication on PIPN.

Since paclitaxel has been described to produce thermal hyperalgesia, TRP channels, that transduce temperature responses, are thought to play a key role on this pathology (Smith et al., 2004). Indeed, previous reports have found that incubation with the TRPV1 antagonist ruthenium red significantly inhibited the thermal hyperalgesia (Hara et al., 2013; Li et al., 2015). In these studies, increased TRPV1 immunoreactivity following paclitaxel administration was also observed. Furthermore, the results were reproducible on human DRG neurons (Li et al., 2015). This alteration has been suggested to be responsible of the burning pain experienced after treatment.

Other TRP channels that might be involved are TRPA1 and TRPM8. The role of these channels on CIPN has not been clearly determined, with few studies relating TRPA1 or TRPM8 with the cold and mechanical allodynia evoked by paclitaxel. On the one hand, TRPA1 antagonist HC-030031 and its knock-out abated cold-allodynia and partially mechanical allodynia (Materazzi et al., 2012). On the other hand, the use of the TRPM8 specific antagonist AMTB reduced the cold-hyperalgesia and tactile-allodynia exhibited by paclitaxel treated mice (Sałat & Filipek, 2015).

Due to the multifactorial components involved in animal studies, there are numerous difficulties for investigating paclitaxel effect. To have more information on the direct exposure of paclitaxel to the peripheral pathway, here, we used a long-term *in vitro* culture of DRG neurons (10 DIV) to investigate the time course of paclitaxel-induced sensitization on neural excitability. For that

purpose, we investigated its functional effect and alterations on Na_V, K_V, TRPV1, TRPA1 and TRPM8 channels. Furthermore, the possible sexual dimorphism in the response was assessed.

Oxaliplatin

Oxaliplatin is one of the most common chemotherapeutics prescribed in cancer treatment. At present, it is the only platinum derivative with activity against advanced colorectal cancer, the fourth most common cancer type with more than a million of cases around the world (Rawla et al., 2019). It is also indicated for the treatment of several digestive tract tumors such as stomach, liver, pancreatic, and esophageal (Zajączkowska et al., 2019). However, it is also one of the most neurotoxic chemotherapeutic agents.

According to clinical data, 30% of colorectal cancer patients stop its treatment due to the oxaliplatin induced-peripheral neuropathy (OIPN) developed (Park et al., 2011). OIPN occurs in 80-96% of patients and became chronic in 40-93%, depending on the dose schedule administered (Argyriou et al., 2013; Ewertz et al., 2015; Yang et al., 2021).

For those patients, OIPN symptoms have the peculiarity of starting acutely as a cold allodynia with cramps, distal and perioral paresthesia, and dysesthesia, that usually start with oxaliplatin injection and can last one week (Cersosimo, 2005). This acute syndrome can also be followed by chronic neurotoxic symptoms that develop with cumulative doses and can last for several years (Beijers et al., 2014; Briani et al., 2014). This chronic neuropathy is characterized by pain, numbness, and dysesthesia (Tofthagen et al., 2013). In this case, mechanical and cold allodynia are the main characteristics of the OIPN symptoms suffered.

At present, there are several mechanisms that have been attributed to the development of oxaliplatin-induced neuropathy including DNA damage, neuroinflammation, mitochondrial damage, oxidative stress, glial activation, and ion channel dysregulation (Descoeur et al., 2011; Kang et al., 2021).

Molecular mechanism of OIPN

Mechanisms of oxaliplatin-induced neuropathy are thought to differ between the acute and chronic forms. For the acute phase, the neuropathy is presumed to arise from the peripheral nerve hyperexcitability caused by the interference with the activity of different ion channels (Wilson et al., 2002). Meanwhile, the chronic phase appears to be the result of complex structural and functional changes such as DNA disruption, mitotoxicity, impaired cellular metabolism, neuroinflammation, and interference with ion channel functionality (Yang et al., 2021). The disruptive effect on the DNA can be

explained because the chemotherapeutic potential of oxaliplatin is based on its ability to form DNA adducts. This interaction can inhibit the DNA replication and transcription leading to cellular apoptosis (Saif & Reardon, 2005; Zajączkowska et al., 2019). In DRG neurons, this ability to form DNA adducts has been correlated with oxaliplatin neurotoxicity in preclinical studies (Ta et al., 2006). These adducts can be formed in the nuclear and mitochondrial DNA (mtDNA) affecting the DNA replication or RNA transcription processing and impairing mitochondrial function (Kang et al., 2021). As a result, decreased mitochondrial respiration and ATP production occur in the sensory neurons. These processes lead to increased oxidative stress and production of reactive oxygen species (ROS) that can further contribute to DNA and mtDNA disruption (Burgess et al., 2021). The subsequent activation of astrocytes releases pro-inflammatory mediators (IL-1β, TNF-α) and diminishes the expression of neuroprotective cytokines (IL-10, IL-4). Then, leucocytes activation drives to a neuroinflammation state

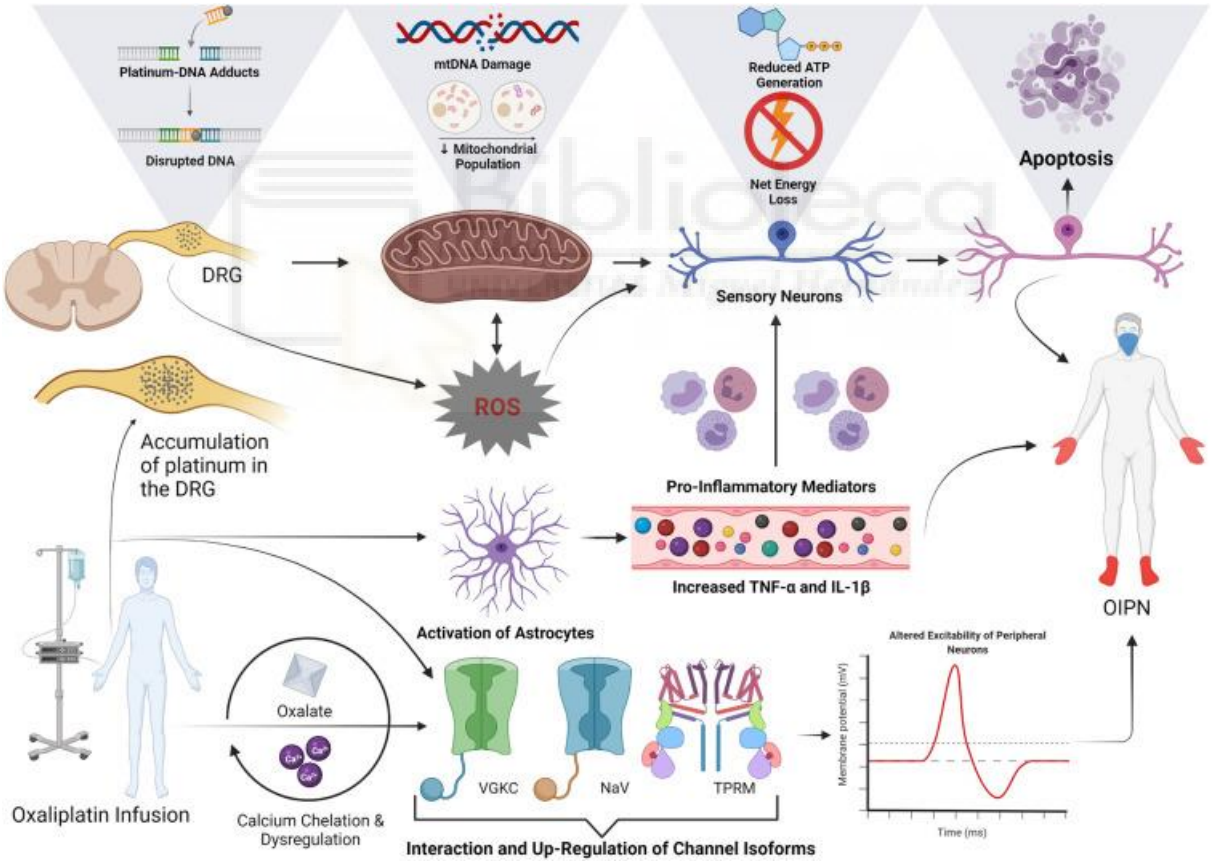


Figure 7. Current hypothesis of the pathogenesis of OIPN. Oxaliplatin infusion results in altered DNA replication and transcription, DNA disruption, mitochondrial damage, reduced ATP production, generation of oxidative stress, neuroinflammation, some neuronal apoptosis and altered ion channel activities. Consequently, nociceptor sensitization evolves leading to OIPN symptoms. VGKC: voltage-gated potassium channels. Figure extracted from Burgess et al. (2021).

on the DRG neurons. The neuroinflammation and oxidative stress can affect the well-being of the neurons leading to apoptosis in some of them. This processes also result in altered ion channel functioning sensitizing nociceptors activity. In addition, oxaliplatin can directly interact with Na_V, K_V, and TRP ion channels causing a major hyperexcitability of the neurons. The oxaliplatin metabolite oxalate has also been described to chelate Ca^{+2} ions in the acute neuropathy phase increasing neuronal excitability (Burgess et al., 2021). A summary of the current hypothesis regarding the molecular mechanisms involved on OIPN is displayed in Figure 7.

As explained, all these changes result in DRG neuronal hyperexcitability through the direct or indirect alterations of different ion channels. This nerve hyperexcitability has been considered as the main contributor to oxaliplatin neurotoxicity (Yang et al., 2021). Accordingly, similar to paclitaxel, Na_V, K_V and TRP channels are emerging as major determinants of oxaliplatin, although considerable differences in the specific mechanisms have been noted between both chemotherapeutics.

In the case of oxaliplatin, the characteristic mechanical and cold allodynia has been attributed to TRPA1 upregulation (Chukyo et al., 2018; Nassini et al., 2011). However, transient changes on TRPM8 have also been observed within hours of oxaliplatin injection and have been hypothesized to contribute to the acute cold allodynia experienced by the patients (Rimola et al., 2021). Other researchers on the contrary neglected the TRP role in oxaliplatin-induced cold allodynia and pointed out to a $Na_V1.6$ dysfunction (Deuis et al., 2013; Sittl et al., 2012).

Other works have also shown increased expression and sensitization of TRPV1 several days following oxaliplatin treatment in parallel with TRPA1 upregulation (Anand et al., 2010; Chukyo et al., 2018). This TRPV1 alteration has been attributed in some extent to the neuropathic pain developed in OIPN but its role has not been clearly determined (Chukyo et al., 2018).

As mentioned before, Na_V channels have also been linked to oxaliplatin-induced neuropathic pain, although there is still a debate on the specific subunits involved. A special focus has been given to $Na_V1.6$ because its presence in large size DRG neurons was essential for the enhanced cooling sensitivity and longer Na_V currents were detected after oxaliplatin treatment (Deuis et al., 2013; Sittl et al., 2012). Apart from this channel, contradictory results have been obtained with $Na_V1.7$ and $Na_V1.8$. Blocking $Na_V1.7$ has been shown to reduce the pain threshold and the hyperalgesia produced by oxaliplatin (Ghelardini et al., 2010). However, the finding that $Na_V1.7$ mutants developed mechanical and cold allodynia normally casts serious doubts on it (Minett et al., 2014). Descoeur et al. (2011) reported that oxaliplatin treatment increased $Na_V1.8$ expression but similar cold and

mechanical allodynia were found in response to oxaliplatin treatment in wild-type and $Na_V1.8$ KO by Minett et al. (2014).

Other pro-excitatory channels such as the hyperpolarization-activated HCN1 protein have also been observed to be down-regulated by oxaliplatin (Descoeur et al., 2011). Indeed, incubation with the HCN blocker ivabradine reversed the oxaliplatin cold hypersensitivity in mice (Descoeur et al., 2011).

In addition, downregulation of the silencing two-pore potassium ion channels TREK1 and TRAAK, and the voltage-gated $K_V1.1$ by oxaliplatin exposure has already been published (Descoeur et al., 2011). These channels are also important mechanical and thermal sensors, being activated by mechanical stimuli and showing decreased functionality with lower temperatures (Maingret et al., 1999; Maingret et al., 2000; Noël et al., 2009). As a result, they have been linked with oxaliplatin neuropathy since TREK1 and TRAAK double KO failed to show the characteristic cold and mechanical hypersensitivity after its treatment (Descoeur et al., 2011). Intriguingly, Dionisi et al. (2020) reported that oxaliplatin induced acidification caused overexpression of TREK channels. However, this overexpression was time-dependent and was hypothesized to compensate only the acute hyperexcitability (Dionisi et al., 2020).

Overall, many different ion channels have been hypothesized to account for oxaliplatin excitability. As it is possible to observe, the mechanisms responsible of the neuropathy symptoms are complex and not completely understood. Consequently, there are still many unanswered questions regarding the specific contribution of these ion channels on OIPN. To address this issue, the second objective of this doctoral thesis consisted of investigating the direct effect of oxaliplatin exposure to the peripheral sensory neurons.

Sex differences in pain

At present, there are numerous pain disorders with a clear sexual dimorphism. Most of the chronic pain conditions have a higher prevalence in women: they constitute approximately 90 % of fibromyalgia patients, 70 % of migraine patients and, near 60 % of people suffering knee and hip osteoarthritis (Fernandez-de-Las-Penas et al., 2010; Jimenez-Trujillo et al., 2019; Mas et al., 2008; Plotnikoff et al., 2015). But the evidence of correlation of sex and pain go further the incidence of the disease.

In several epidemiological studies, women showed higher pain sensitivity. Indeed, in different experimental models of pain, scientists have detected that the mechanical nociceptive threshold is

reduced in females, implicating that they would detect as painful softer mechanical stimulus than males (Aloisi et al., 1994; Bourquin et al., 2006; Cook & Nickerson, 2005).

Furthermore, pain could be experienced with more severity for women. When people were asked to rate their pain intensity, women pointed out bigger scores for similar pain-related disorders than men (Bingefors & Isacson, 2004; Rustoen et al., 2004). Regarding the response to an inflammatory environment, in the Complete Freund's Adjuvant (CFA) model, females showed reduced mechanical rheobase than male (Cook & Nickerson, 2005). Similarly, higher osteoarthritis severity was experienced more frequently in broader areas of the body by women (Srikanth et al., 2005), whereas men showed higher pain threshold in all the tender point (Segura-Jimenez et al., 2016). For neuropathic pain, stronger mechanical allodynia was measured in female mice following spinal nerve transection (DeLeo & Rutkowski, 2000; Melchior et al., 2016).

Despite the increasing evidence of a sexual dimorphism in pain pathways, clinically it has been poorly addressed. This could be partially explained because the majority of the scientific literature has conducted their experiments on male, extrapolating these results to females and ignoring the possible sex differences that may exist (Lauretta et al., 2018). It is estimated that almost 80 % of the studies of pain were conducted on male subjects and only 4 % of them would have compared both sexes (Mogil & Chanda, 2005). This paradox could also explain why numerous analgesics developed result more effective in men than in women. Noteworthy, in 2005, 8/10 prescription drugs were withdrawn from the US market due to women's health issues (Lauretta et al., 2018).

Thus, in the last years, studying the role of sex on pain behavior has emerged as a key research topic (Cabañero et al., 2022; Clayton & Collins, 2014). Knowing better how pain pathways are modulated in males and females would open a novel venue for a satisfactory and personalized treatment of pain.

Sex differences in CIPN

For CIPN, clinical and pre-clinical studies are providing evidence on the presence of sexual dimorphism in its pathophysiology (Davidson et al., 2019; Ferrari et al., 2020; Villalba-Riquelme et al., 2022; Wagner et al., 2021). Since the therapeutic window of chemotherapeutic drugs is very narrow, special concerns have been made regarding this potential difference (Wang & Huang, 2007). Small differences on its pathophysiology would result in increased toxicity, thus compromising patients' survival and quality of life. Therefore, investigating the potential sex differences in CIPN would be crucial for designing a more personalized treatment. With this aim, as part of the present doctoral thesis, we performed a bibliographic search of clinical and pre-clinical studies that analyzed CIPN in

female and male subjects. The results found for the different chemotherapeutic treatments are summarized in Table 2.

Table 2. Sex differences found in CIPN neuropathy and pain symptoms after treatment with different chemotherapeutic drugs in clinical and pre-clinical studies.

Chemotherapeutic Drug	Potential thermoTRPs Involved	Sex differences observed	Reference
Taxanes			
Paclitaxel	TRPV1 TRPM8 TRPV4 TRPA1	Reduced rheobase, higher sensitivity in female rat DRG neurons exposed to paclitaxel Cold allodynia more robust in female mice Greater magnitude of paclitaxel hyperalgesia in female rats Higher TRPM8 expression in male rat DRG neurons Mechanical pain produced through estrogen dependent IL-23/IL-13/TRPV1 signaling axis only in female mice No significant sex difference in the response to mechanical stimuli for male and female rats	(Villalba-Riquelme et al., 2022) (Naji-Esfahani et al., 2016) (Ferrari et al., 2020) (Villalba-Riquelme et al., 2022) (Luo et al., 2021) (Hwang et al., 2012)
Docetaxel	TRPV1	Not investigated alone	
Platinum-based compounds			
Oxaliplatin	TRPV1 TRPM8 TRPA1	Reduction in nerve conduction amplitude in female mice	(Warncke et al., 2021)
Cisplatin	TRPV1 TRPV2 TRPA1 TRPM8	More persistent tactile allodynia in male than in female mice Bigger incidence of prolonged heat latency in male rats	(Woller et al., 2015) (Wongtawatchai et al., 2009)
Carboplatin	TRPA1	Not investigated alone	
Vinca-alkaloids			

Vincristine	TRPV1 TRPV4	Mechanical hyperalgesia was higher in female than in male rats Statistically significant reduced mechanical sensitivity threshold only in male rats	(Joseph & Levine, 2003) (Legakis et al., 2020)
Proteasome inhibitor			
Bortezomib	TRPA1 TRPV1	The prevalence of peripheral neuropathy was nearly double in women treated with bortezomib (65.3%) compared to men (36%) (retrospective study using clinical data) Significant lower threshold of mechanical sensitivity at day 30 in female but not in male Sprague-Dawley rats S1PR1 antagonists prevented bortezomib mechano-allodynia and mechano-hyperalgesia in male but not in female rats Male sex was a predictor of bortezomib-induced CIPN development (retrospective analysis using clinical data)	(Martinez et al., 2019) (Legakis et al., 2020) (Stockstill et al., 2020) (Kanbayashi et al., 2010)
Alkylating agents			
Ifosfamide	TRPA1?	Higher neurotoxicity in females	(Schmidt et al., 2001)
Antibody-Drug Conjugates (ADC)			
Brentuximab-vedotin Enfortumab-vedotin	Not investigated	Not disclosed, but likely similar to vincristine as auristatin E as both share a similar inhibitory mechanism	
Bevacizumab	Not investigated	More abdominal pain in females than males with advanced-stage non-small cell lung cancer (clinical trials)	(Brahmer et al., 2011)
Hormonal therapies: Aromatase inhibitors			
Anastrozole	TRPA1	Not studied in male (breast cancer)	
Immunomodulatory drugs			
Thalidomide	TRPA1 TRPV4	Not investigated	
Antimetabolites			
5-Fluorouracil (Capecitabine)	Not investigated	Female patients with advanced colorectal cancer had significantly higher risk for hand-foot syndrome (clinical trials)	(Lévy et al., 1998)
Epothilones			
Ixabepilone	Not investigated	Not studied in male, only in female	
Combination of therapies			

leucovorin + fluorouracil + oxaliplatin (FOLFOX)	Not investigated	Higher incidence of peripheral neuropathy and hand-foot syndrome in female patients with colorectal cancer (clinical trials) (Wagner et al., 2021)	(Wagner et al., 2021)
Combined (not mentioned)	Not investigated	Increased I-III grade neurological toxicities in female patients (clinical trials database, *N* = 23256 patients)	(Unger et al., 2022)
Combined	Not investigated	Female sex associated with higher neuropathy sum score after treatment of Hodgkin's lymphoma (clinical trials)	(Eikeland et al., 2021)
Combined (ECF, ECX, EOF or EOX)	Not investigated	Males showed higher incidence of all-grade peripheral neuropathy in oesophagogastric cancer	(Davidson et al., 2019)
FOLFOX/ bevacizumab	Not investigated	No differences for grade ≥ 3 sensory neuropathies in patients with unresectable advanced or recurrent metastatic colorectal cancer	(Yamada et al., 2020)
Paclitaxel and/or Oxaliplatin	Not disclosed, but likely TRPV1, TRPA1 and TRPM8	Female associated with higher neuropathy score (cohort study of 333 patients)	(Mizrahi et al., 2021)
Combination of PC, CG, CD or CaP	Not investigated	Tendency in women with advanced non-small cell lung cancer to have more neurosensory deficits than men (clinical trials)	(Wakelee et al., 2006)

Table extracted from the review included in this thesis (Cabañero et al., 2022) (Publication 2). E: Epirubicin; C: Cisplatin; F: Fluorouracil; X: Capecitabine; O: Oxaliplatin. L: Leucovorin. G: Gemcitabine. D: Docetaxel. Ca: Carboplatin. P: Paclitaxel.

As we can observe in Table 2, in most of the clinical findings, women experienced higher severity, incidence and/or sensitivity of the peripheral neuropathy symptoms (Cabañero et al., 2022; Lévy et al., 1998; Mols et al., 2016; Trendowski et al., 2021; Unger et al., 2022). These results may vary between chemotherapeutic agents, however, poor information regarding individual chemotherapeutic drugs is reported since combination of chemotherapeutic regimens is usually prescribed in the clinical practice.

For paclitaxel and/or oxaliplatin treatment, Mizrahi et al. (2021) showed that female sex was associated with higher neuropathy score in a cohort study of 333 patients. These sex differences were also observed in pre-clinical studies. In agreement with these results, in our first publication from this

doctoral thesis, we found higher sensitivity and reduced rheobase in female rat DRG neurons after paclitaxel treatment (Villalba-Riquelme et al., 2022). Other studies also found greater magnitude of paclitaxel hyperalgesia, more robust cold allodynia, and mechanical pain in female rats (Ferrari et al., 2020; Luo et al., 2021; Naji-Esfahani et al., 2016). For oxaliplatin regimen, we could find only one article addressing these differences from Warncke et al. (2021) that showed reduction in nerve conduction amplitude only in female mice.

In addition, sex differences in CIPN pharmacology have also been described (Ram et al., 2021), suggesting that adaptation of the therapeutic strategy to the patient sex appears an important factor for a satisfactory disease management. In support of this tenet, promising results were obtained when the chemotherapeutic dose was specifically adapted to each sex and age (Pfreundschuh et al., 2017). Therefore, investigating the molecular mechanisms underlying the pathophysiology of CIPN in males and females would be essential for developing more efficient pharmacological tools.

Potential molecular mechanisms of CIPN sex differences

Studying the pain pathways for male and female subjects has unrevealed important differences on its molecular mechanisms (Melchior et al., 2016). Notably, novel research is driving to a key role of TRP channels in CIPN sexual dimorphism (Luo et al., 2021; Villalba-Riquelme et al., 2022). Thus, as part of this doctoral thesis, we also reviewed the advances on this topic and evaluated the potential molecular mechanisms that could explain this sexual dimorphism (Cabañero et al., 2022) (Publication 2). This information has been summarized in the corresponding Results section and compiled in Figure 8.

Sex differences in TRPV1 axis pathway have just been described in response to paclitaxel treatment (Luo et al., 2021). Luo et al. (2021) detected a specific mechanical pain driven mechanism through IL-23/IL-17A/TRPV1 axis only in female mice. This mechanism was considered responsible of the higher mechanical pain detected in female (Luo et al., 2021). In our study, we could also find that female neurons were more sensitive to paclitaxel treatment (Villalba-Riquelme et al., 2022). However, TRPV1 activity similarly augmented in male and female neurons. Interestingly, the TRPM8 mediated currents of the paclitaxel group were higher in male than in female sensory neurons (Villalba-Riquelme et al., 2022).

In addition, TRP channels have been found to be modulated by sex hormones and this communication has been considered a key underlying mechanism of sexual dimorphism in chronic pain (Artero-Morales et al., 2018; Cabañero et al., 2022). It is well established that one of the major physiological differences between men and women remain in the presence, quantity, and fluctuations

of sex hormones (Caruso et al., 2013). Measurements of the levels of gonadal hormones in the peripheral nervous system of male and female rats resulted in increased testosterone levels in male rats, whereas 17-β-estradiol and progesterone derivatives were more abundant in female rats (Caruso et al., 2013). Moreover, it has been reported that pain sensation can vary between the women menstrual phases. During these phases, the levels of two of the main female hormones estrogen and progesterone experiment high fluctuations. For example, migraine attacks are more frequently produced in the late luteal phase/early follicular phases of the menstrual cycles coinciding with lower estrogen levels (MacGregor et al., 2006).

In the case of CIPN, estrogens have been suggested to act as proalgesic agents in females through TRP pathways by several studies. For the chemotherapeutic vincristine, inhibition of PKC-ε, a modulator of TRPV1 activity, diminished the induced hyperalgesia only in males and ovariectomized females (Joseph & Levine, 2003), presuming that estrogens could be promoting TRPV1 signaling to generate this hyperalgesia (Goswami et al., 2011). In support of this tenet, in paclitaxel CIPN, the IL-23/IL-17/TRPV1 axis was activated by estrogens to induce mechanical pain in female rats (Luo et al., 2021). Similarly, Wang et al. (2018) described that ovariectomized rats exposed to paclitaxel had a lower pain response. There are also other studies pointing towards a protective role of 17-β-estradiol against CIPN symptoms (Miyamoto et al., 2021), however most of the research suggests that estrogen antagonists could provide pain relieve (Paller et al., 2009).

On the contrary, progesterone and its derivatives have been considered to protect against CIPN (Falvo et al., 2020). Progesterone diminished the neurotoxicity caused by cisplatin (Zaki et al., 2018), prevented the neuropathy in docetaxel treated rats (Roglio et al., 2009), and had antinociceptive effects on vincristine treated rats (Meyer et al., 2010). In addition, allopregnanolone, a progesterone derivative, was capable of suppressing oxaliplatin neuropathy (Meyer et al., 2010). As an explanatory mechanism, the progesterone downregulation of TRPV1, TRPA1 and TRPV4 has been previously linked with this protective effect (Jung et al., 2009; Ortíz-Rentería et al., 2018) (Figure 8).

Similarly, testosterone has been proposed as having antinociceptive effects in chronic pain (Alarcón-Alarcón et al., 2022; Roglio et al., 2007; Tanzer & Jones, 2004). This hormone was able to reduce TRPV1 expression in a mouse inflammatory pain model (Bai et al., 2018) and is considered as a direct TRPM8 agonist (Asuthkar et al., 2015). This neuroprotective effect together with the higher levels of this hormone found in male animals, suggests that testosterone could have a primordial role in the sexual dimorphism of chronic pain disorders (Alarcón-Alarcón et al., 2022; Roglio et al., 2007). Despite these observations, to my knowledge, no studies have been directed to investigate its contribution in CIPN. Nevertheless, several chemotherapeutic agents such as cisplatin and docetaxel

inhibited testosterone synthesis diminishing its levels (García et al., 2012; Ryan et al., 2020). Thus, for the complete understanding of sexual dimorphism in CIPN, there is a need to investigate the role of testosterone on it.

In addition, sex differences have been found in the immune toll-like receptors signaling (TLR) through modulation of TRP channels (Luo et al., 2019). The use of a TLR9 antagonist attenuated the mechanical pain evoked by paclitaxel treatment only in male mice and this alteration was mediated by promoting TRPV1 functionality (Luo et al., 2019). Sorge et al. (2011) also observed that TLR4 mediated inflammatory and neuropathic pain responses in male but was absent in female mice. TLR4 also contributed to the development of paclitaxel neuropathy through TRPV1, and cisplatin and carboplatin were described as ligands of the TLR4 receptor (Li et al., 2015; Park et al., 2014).

Apart from the TRP implication, other physiological differences such as increased epidermal nerve fiber density in females, sexual dimorphism in immune and glial mediated response, and pharmacokinetics of the drugs have also been noticed in the literature (Fitzgerald & Salter, 2019; Gregus et al., 2021; Lévy et al., 1998).

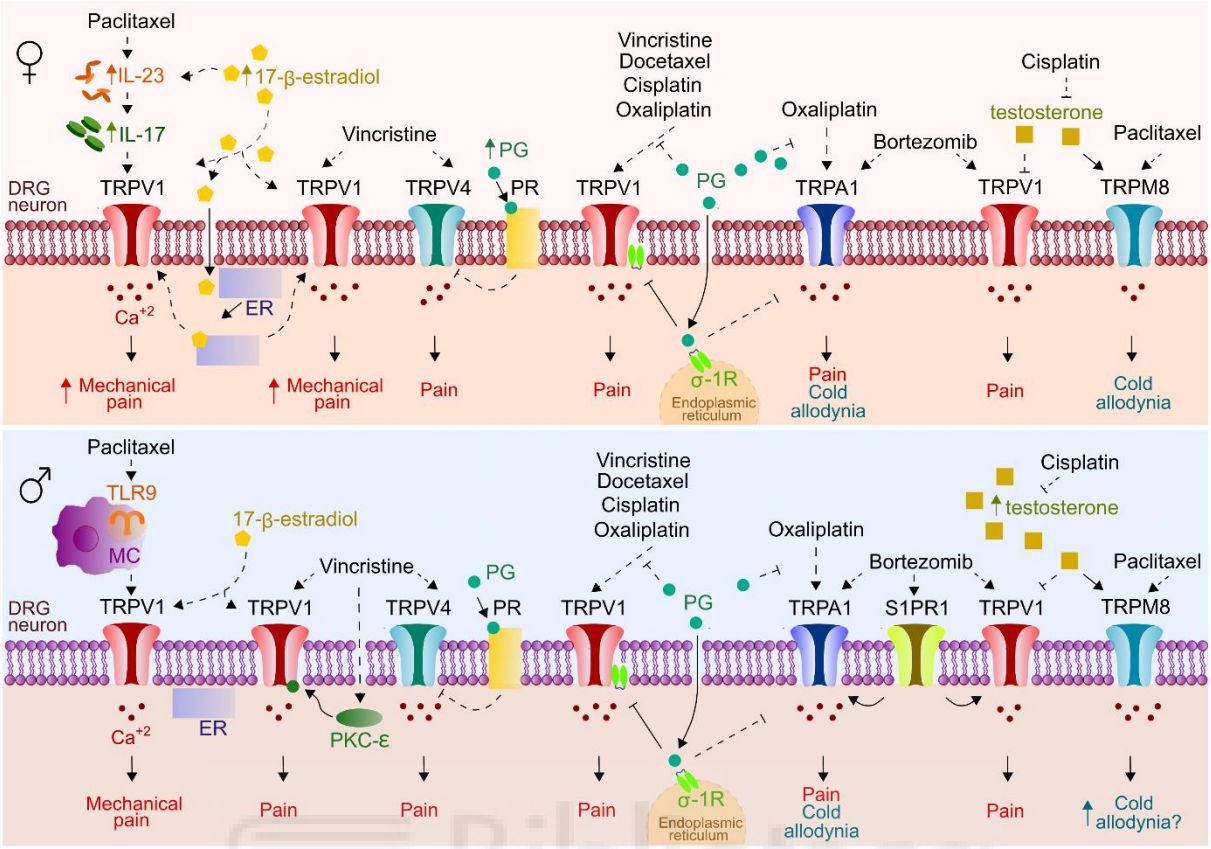


Figure 8. Schematic representation of potential mechanisms underlying sexual dimorphism on CIPN. Paclitaxel induced mechanical pain was produced through IL-23/IL-17/TRPV1 signaling only in female mice (Luo et al., 2021). The IL-23 induced pain was promoted by the sex hormone 17-β-estradiol. 17-β-Estradiol also showed a pivotal role on vincristine-induced neuropathy symptoms, since removal of this hormone reduced the greater mechanical hypersensitivity found in female rats compared to male (Joseph & Levine, 2003). As a major mechanism, 17-β-estradiol was found to increase TRPV1 expression through its binding to estrogen receptor (ER) (Payrits et al., 2017). In contrast, progesterone showed a protective role in the neuropathy induced by cisplatin, docetaxel, vincristine and oxaliplatin (Meyer et al., 2010; Roglio et al., 2009; Zaki et al., 2018). The reduction of pain behavior produced by progesterone could be the result of decreased TRPV1 levels and/or reduced TRPA1 activity through inhibition of σ-1R receptor or, in the case of vincristine, it could also be due to diminished expression of TRPV4 through binding to its progesterone receptor (Jung et al., 2009; Marcotti et al., 2023; Ortíz-Rentería et al., 2018). As male specific mechanisms, paclitaxel mechanical pain was modulated by TLR9 only in male, possibly through alteration of TRPV1 channel activity (Luo et al., 2019). Furthermore, higher TRPM8 responses after paclitaxel exposure were observed on male DRG neuronal cultures (Villalba-Riquelme et al., 2022). TRPM8 has also been described as a testosterone receptor (Asuthkar et al., 2015). Testosterone was also postulated to decrease pain by reducing TRPV1 expression (Bai et al., 2018). S1PR1 antagonists only prevented neuropathic pain on male rats (Stockstill et al., 2020). There, S1PR1 could evoke pain through TRPA1 and TRPV1 channels (Kittaka et al., 2020). Inhibition of PKC-ε, a modulator of TRPV1 activity through phosphorylation, only reduced vincristine-induced hyperalgesia in males and ovariectomized females (Joseph & Levine, 2003). Dashed lines indicating indirect interaction. Figure extracted from Publication 2 of this doctoral thesis (Cabañero et al., 2022).

As a result, there is an urgent need that pre-clinical studies evaluate the role of sex in CIPN disorder. Despite this evidence, the presence of sexual dimorphism and its potential molecular mechanism in paclitaxel and oxaliplatin remains poorly addressed. To address this issue, in the present doctoral thesis we compared and analyzed the differences in the response of male and female rat sensory neurons to the chemotherapeutic agents' paclitaxel and oxaliplatin.

To start with this research, we first needed to select a translational system for investigating pain mechanisms. Here, the three major groups used for pain research are summarized.

How do we study pain?

Classically, for studying pain the main options taken were: investigating it on patients and healthy volunteers or using animal models. However, these studies present numerous limitations.

Patients and Healthy volunteers

On the one hand, patients are the most translational group since it allows to investigate the pain in its pathologic environment in humans. However, due to the stronger ethical concerns and technical limitations, they can be included only in few and very restricted studies. Thus, investigating pain in humans can only be performed with non-invasive techniques like questionaries, quantitative sensory testing, conditioned pain modulation, neuroimaging and/or analyzing biomarkers from urine, blood samples or biopsies (Mouraux et al., 2021) (Figure 9). But these techniques do not reveal the structure and function of the pathology at the cellular level.

Similarly, research on healthy human volunteers can also be explored with the same arsenal of techniques (Mouraux et al., 2021). In this case, the information obtained can be used to understand general pain mechanisms and behavior rather than specific pain conditions. However, the same limitations than in patients research arise. Furthermore, sampling bias have been noted, indicating that people with certain psychological characteristics, such as less fear to pain, participated in these studies, thus reducing extrapolation of the findings to the whole population (Karos et al., 2018).

Animal models

On the other hand, animal models have provided valuable information on pain processes since the 19th century (Mouraux et al., 2021). One of the most interesting aspects of this research is that animals are susceptible of the same pain pathologies occurring in humans, providing very useful

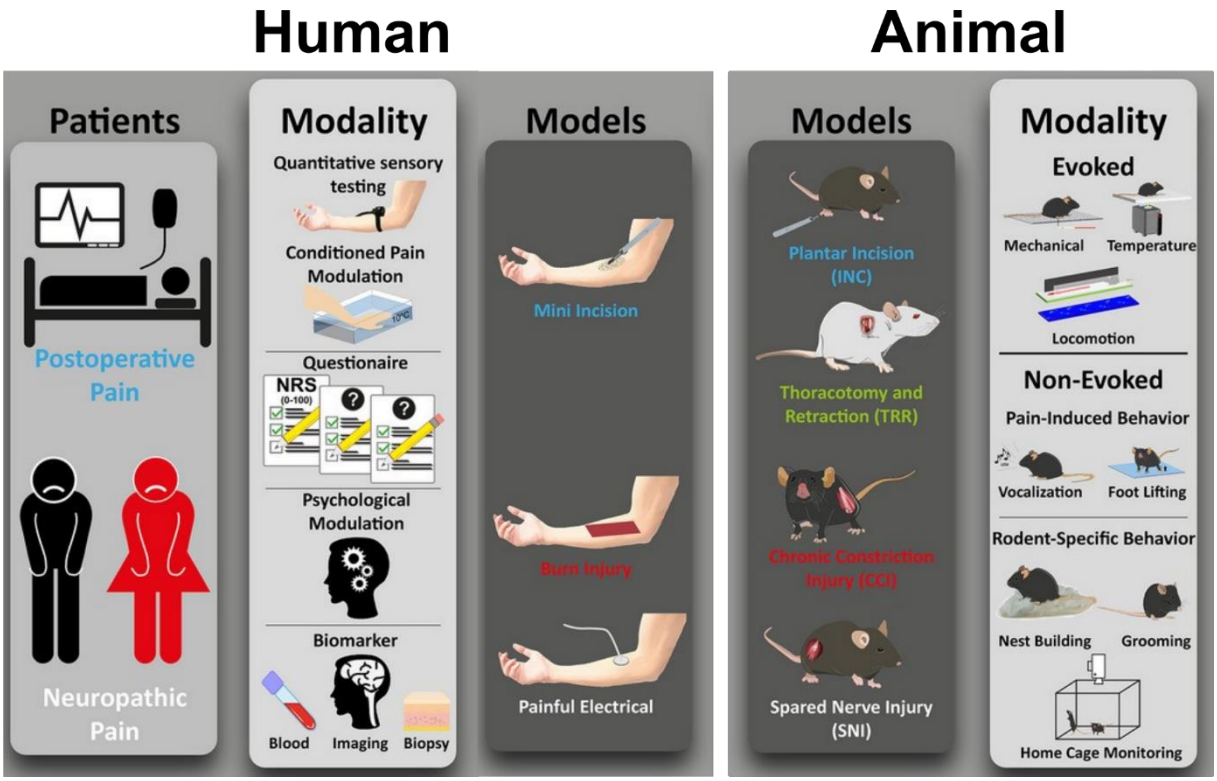


Figure 9. Classical pain research was based on patients, experimental human models, and animal models of pain. Figure modified from the article of Mouraux et al. (2021).

models for a specific pathophysiological condition (Mogil et al., 2010). Furthermore, they are necessary for the pharmacological evaluation of new analgesics, since they are key systems to predict the toxicity and efficacy of these drugs (Mogil et al., 2010). However, there are numerous challenges and difficulties when dealing with animal models.

First, the increasing ethical concerns related to animal models minimize the quantity of subjects that can be studied compromising the possibility to generate solid data and statistically analyzing it (Denayer et al., 2014; Mogil, 2009). Second, the validity of the translation of these studies is questioned: the measurement of subjective outcomes and the lack of consideration of many modulatory pain factors on these models leads to the fact that the drugs success during clinical development remains poor (Denayer et al., 2014; Mogil, 2009). Third, inter-species variability limits the extrapolation of the findings to humans. Consequently, since pain is a burden health problem, we need supporting tools for studying it.

In vitro models

In the last years, *in vitro* cultures have started to gain attention as powerful tools for investigating pain (Chrysostomidou et al., 2021; Villalba-Riquelme et al., 2022). They allow scientists to control the environment and simplify potential interfering variables, resulting in a more detailed study of the molecular pathways. Their lower associated costs and ethical concerns, together with their easier manipulation, are presumed to facilitate and accelerate pain research. Due to these reasons, the number of samples that can be used is much higher than in the previous approaches, offering more quantitative and consistent data. These interesting characteristics have exponentially raised its application in research such as in drug screening assays where a large number of conditions need to be tested (Chen et al., 2011; Chrysostomidou et al., 2021).

These interesting characteristics have been translated in an exponential increase in the use of *in vitro* cultures in different modalities. At present, the cell types that can be used to study peripheral sensory neurons include human DRG neurons, stem cell-derived sensory neurons, and DRG neurons extracted from animals (Chrysostomidou et al., 2021). The use of **human DRG neurons (hDRG)** avoids the inter-species variability produced by animal models and provides detailed information on pathological molecular mechanisms (Chrysostomidou et al., 2021). However, access to these types of cells is extremely restricted by its low availability and they couldn't be used in this study. Differences in the donor characteristics might also impact the consistency of the results leading to greater variability between samples (Chrysostomidou et al., 2021).

Stem-cell derived sensory neurons (SC-SN) have been generated to reduce the dependence on the use of humans and animals, thus increasing its availability for research. They can be generated from embryonic stem cells (hESCs) (Young et al., 2014) or induced pluripotent stem cells (iPSCs) (Chambers et al., 2012), being the latest the more commonly used. iPSCs allows the sample obtention from human source with non-invasive procedures as the extraction of skin fibroblasts (Takahashi & Yamanaka, 2006). The most common applications for iPSCs are the investigation of the effect of specific mutations linked to pain disorders and analgesics screening (Ebert et al., 2012; McDermott et al., 2019). These interesting characteristics are suggesting an exponential increase of the use of iPSCs in future research. However, nowadays, differentiation protocols have not achieved to obtain the full diversity of DRG neurons present in complex organisms (Chrysostomidou et al., 2021; Sharma et al., 2020).

Thus, for *in vitro* cultures, the use of **rodent DRG neurons** represents the current most common approach. Nevertheless, most of these cultures are short-term just to adapt them to the quantitative measuring techniques requirements (e.g. patch clamp, MEA), performing the treatment

in the living animals (Dionisi et al., 2020; Zhang & Dougherty, 2014). This implicates stronger ethical concerns that restrict these studies, especially in the field of pain, where this unpleasant sensation needs to be induced in the animals (Alarcón-Alarcón et al., 2022; Bourquin et al., 2006; Descoeur et al., 2011; Luo et al., 2019; Su et al., 2021). Furthermore, the invasive and thorough procedure by which these neurons are extracted together with the time needed to wait before measuring them (e.g. 1-2 days in electrophysiological systems), might interfere with the detection of the pathological alterations developed in the living animals (Leffler et al., 2002; Marcotti et al., 2023; Nguyen et al., 2019). There, the use of long-term cultures of sensory neurons could face these problems.

Long-term cultures of rodent sensory neurons have started to be used in the last years in parallel with the improvement of culturing media conditions (Newberry et al., 2016). Sensory neurons presented a short survival in culture that put up a barrier to the ability to study long-term exposures to algesic agents, the subsequent desensitization process, or the time dependent response to them, as occur in chronic pain conditions (Ponsati et al., 2012; Proudfoot et al., 2006). Extending their survival to have long-term cultures allows us to investigate the time-course and the exact mechanisms of the exposure of the sensory neurons to algesic agents in a controlled environment. This drives to the development of "pain-in-a-dish models" that are emerging to facilitate pain research. In the case of peripheral neuropathies like CIPN, where damage to the peripheral sensory neurons is the main cause of these symptoms, long-term cultures offer a promising strategy for investigating them. Thus, novel research is showing its high translational potential and obtaining promising results with them (Li et al., 2020; Villalba-Riquelme et al., 2022).

Consequently, a growing body of research suggests that developing *in vitro* models of different pain conditions could be crucial for the future of pain research. With this aim, we designed the present doctoral thesis to stablish and study different *in vitro* pre-clinical models of chemotherapy-induced peripheral neuropathy using long-term DRG cell cultures obtained from male and female rats, investigating the underlying functional and molecular mechanisms, and evaluating the presence of sexual dimorphism.

Objectives

Objectives

The present doctoral thesis is aimed to investigate the molecular mechanisms implicated on nociceptors sensitization and desensitization after a long-term exposure to the chemotherapeutic agents' paclitaxel and oxaliplatin and its sexual dimorphism.

To that purpose, the following specific objectives were designed:

1. Understanding the time-course effect of the direct paclitaxel exposure in an *in vitro* preclinical model of the chemotherapy-induced peripheral neuropathy.

2. Elucidating the molecular and functional changes that occur after nociceptors prolonged exposure to oxaliplatin developing an *in vitro* pre-clinical model of the chemotherapy-induced peripheral neuropathy.

3. Characterization of the sexual dimorphism in the nociceptive response to chemotherapeutic agents.

Materials and Methods

Materials and Methods

Animals and ethic statement.

Most of the *in vivo* studies regarding paclitaxel neurotoxic effect have been previously performed on Wistar rats due to the similarities with the human pain pathway (Hara et al., 2013; Y. Li et al., 2018). Therefore, our experiments were conducted on 12-20 weeks old male and female Wistar rats obtained from Servicio de Experimentación Animal (SEA) of Miguel Hernández University of Elche. Animals were housed in polycarbonate plastic cages (2-4 animals per cage) at 21-23 ^{o}C with a 12 h light/dark cycle in a controlled environment with water and food available *ad libitum*. All procedures were approved by the Institutional Animal and Ethical Committee of the Miguel Hernández University of Elche (UMH.IDI.AFM.06.20) and the Autonomous Government of Valencia (2021/VSC/PEA/0089), in accordance with the guidelines of the Economic European Community in accordance with Directive 2010/63/EU, the National Institutes of Health, and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. The experimental protocols are reported in accordance with the ARRIVE 2.0 and the British Journal of Pharmacology guidelines (Lilley et al., 2020; Percie du Sert et al., 2020).

Culture of DRG neurons.

Rats under isoflurane anaesthesia (IsoFlo®, Zoetis) were decapitated and the vertebral column was excised. DRG were then isolated in a Petri dish containing Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) with the use of a stereo microscope (VWR). Thereafter, DRG neurons were isolated incubating the ganglia with 0.25 % (w/v) collagenase type IA (Sigma-Aldrich) in DMEM (Gibco) with 1 % penicillin-streptomycin (P/S, 5000 U·mL^{-1}, Invitrogen) at 37 ^{o}C for 1 h in 5 % CO_2. After digestion, DRG were mechanically dissociated with the pipette, placed into DMEM medium containing 10% of foetal bovine serum (FBS, Invitrogen) and 1% P/S and centrifuged to remove rests of connective tissue. Cell culturing conditions were adapted from a previously described protocol (Newberry et al., 2016). One hour later, cell medium was replaced by Neurobasal-A medium (Gibco) supplemented with 1 % P/S, B-27® Supplement (2 %; Gibco) and GlutaMAX$_{TM}$ (1 %; Invitrogen). Nerve growth factor (NGF, 5 ng·mL^{-1}; Sigma-Aldrich), NaCl (5 mg·mL^{-1}; Sigma-Aldrich), uridine (17.5 µ·mL^{-1}; Sigma-Aldrich) and 5-fluoro-2-deoxyuridine (7.5 µg·mL^{-1}; Sigma-Aldrich) were added to cell medium 24 h after seeding. For microelectrode arrays (MEA), due to technical requirements, the final NGF concentration was 100 ng·ml^{-1}. Cells were maintained at 37 ^{o}C in a humidified incubator with 5 % CO_2. During cell culture,

half of the medium was changed for fresh medium every 3-4 days. For paclitaxel model, five days after cell seeding, cells were treated with 1 µM of paclitaxel (Taxol®, Tocris Bioscience) or with the vehicle (0.04 % DMSO) for 24 h. For oxaliplatin model, two different time conditions were tested: application to cell culture of 1 $\mu g \cdot mL^{-1}$ of oxaliplatin (Oxaliplatin, Tocris Bioscience) or with vehicle (double distilled water, ddH_2O) 4 days after cell seeding for 48 h, or 5 days after cell seeding for 24 h.

Immunofluorescence staining

For immunofluorescence staining, DRG neurons were previously seeded in coverslips. The day of the experiment, the coverslips were washed with DPBS and fixed in 4 % (v/v) paraformaldehyde (Sigma-Aldrich) during 20 minutes at room temperature (RT). To remove paraformaldehyde, three washes with DPBS were performed. Then, permeabilization was performed with 0.1 % Triton X-100 (Sigma-Aldrich) for 5 minutes. After washing the cells with DPBS to prevent non-specific labelling, a blockage step was performed using 5 % normal goat serum (NGS, Sigma-Aldrich) at RT during 1 h. Then, the blocking solution was removed, and the primary antibodies were added diluted in DPBS solution with 5 % NGS. The following primary antibodies were used: a mouse monoclonal antibody against neuron-specific protein NeuN at 1:50 dilution (Millipore, Cat# MAB377C3, RRID:AB_10918200); and a rabbit polyclonal antibody against one of the following targets each time: TRPV1 using at a 1:100 dilution, (Alomone Labs, Cat# ACC-029, RRID:AB_2040258); TRPA1, at 1:100 (Alomone Labs, Cat# ACC-037, RRID:AB_2040232); TRPM8, at 1:100 (Alomone Labs, Cat# ACC-049, RRID:AB_2040254); $Na_V1.7$, at 1:200 (Alomone Labs, Cat# ASC-008, RRID:AB_2040198); $Na_V1.8$, at 1:200 (Alomone Labs, Cat# ASC-016, RRID:AB_2040188); and $Na_V1.9$, at 1:200 (Alomone Labs Cat# ASC-017, RRID:AB_2040200), respectively. These antibodies were incubated overnight at 4 °C with gentle agitation. The following day, cells were washed with DPBS and incubated with goat anti-rabbit IgG Alexa Fluor® 488, 1:500 (Thermo Fisher Scientific, Cat# A-11034, RRID:AB_2576217) and goat anti-mouse IgG Alexa Fluor® 568, 1:500 (Thermo Fisher Scientific, Cat# A-11031, RRID:AB_144696) secondary antibodies at ~22 °C for 1 h. After a DPBS wash, cells were stained with DAPI, 300 nM (Thermo Fisher Scientific Cat# D1306, RRID:AB_2629482). Coverslips were mounted with Mowiol® (Calbiochem). All dilutions were freshly prepared the day of the experiment from original stocks stored in individual aliquots at -20 °C. Samples were visualized with an inverted fluorescence microscope (Zeiss Axio Observer, Carl Zeiss). Fluorescence was quantified removing the mean background fluorescence from each cell fluorescence using Fiji® software (Schindelin et al., 2012). Representative pictures shown were obtained with an inverted confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss).

Patch-Clamp recordings.

Small-diameter (< 30 µm) DRG neurons seeded in coverslips were registered in voltage and current-clamp modes. For cell culturing, crystals were coated with poly-L-lysine (50 $\mu g \cdot mL^{-1}$, Sigma-Aldrich) for 2 h. After four washes with deionized water, crystals were incubated during 1 h at 37 ^{o}C with laminin diluted in DMEM medium (10 $\mu g \cdot mL^{-1}$; Sigma-Aldrich). After DRG neurons extraction, laminin was replaced by the cell suspension diluted in DMEM 1 % FBS 1 % P/S. Patch pipettes from borosilicate glass with OD 1.5 mm x ID 1.17 mm (Warner Instruments) were pulled using a Flaming/Brown micropipette puller P-97 (Sutter Instruments) to have 2-5 MΩ resistance. Seal-resistance was between 200 MΩ and 1.5 GΩ and series resistance was compensated around 80 %. Extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 Glucose, 20 Mannitol, pH 7.4 adjusted with NaOH. Pipette internal solution contained (in mM): 144 KCl, 2 $MgCl_2$, 10 HEPES, 5 EGTA, pH 7.2 adjusted with KOH. Experiments were performed in whole-cell configuration at ~ 22 °C. After stablishing whole-cell access, cells were recorded in current-clamp mode. First, the resting membrane potential (RMP) was determined without any current injection. Cells with RMP higher than -40 mV or AP that did not overshoot 0 mV were not considered for analysis. Neurons that fired action potentials in the absence of stimulus were considered as having spontaneous activity (SA). To calculate the rheobase, the firing frequency, and to classify neurons into tonic or phasic behaviour, 1 s-current depolarizing pulses from 0 to 300 pA in 10 pA intervals were applied. The minimum current required to evoke the first action potential was considered as the current rheobase. Neurons were classified as phasic if they fire one or few action potentials at the onset of the current stimulus. Tonic neurons were those that were able to fire continuously during one or more of the 1 s current pulses. The AP parameters were measured in the action potential fired at the minimum current injected using 10 ms depolarizing pulses from 0 to 300 pA in 10 pA steps. AP threshold was considered when the upstroke slope was ≥10 $V \cdot s^{-1}$. AP amplitude was measured from RMP to peak.

For voltage-clamp recordings, capacitive transients were compensated. Cells with capacitance values higher than 40 pF were excluded from the analysis. For measuring K^+ currents, the fast-inactivating K^+ current (K_A) and the non-inactivating K^+ current remaining before the end of the protocol (K_{DR}), respectively, were measured in a 300 ms voltage-step protocol from -80 to 70 mV in 10 mV intervals. For registering Na^+ currents, external solution contained (in mM): 70 NaCl, 65 Choline Chloride, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 TEA-Cl, $CsCl_2$ 10 HEPES and 10 Glucose, pH 7.4 adjusted with NaOH. Pipette internal solution contained (in mM): 140 CsF, 10 NaCl, 1 EGTA, 5 Glucose and 10 HEPES, pH 7.30 adjusted with CsOH. $Na_V1.8$ currents were isolated using previous described voltage steps protocols (Soriano et al., 2019), The G-V curves were calculated from current-voltage relations, using $G=I_x/(V-V_x)$, where I_x and V_x are the ionic currents and the equilibrium potential for Na^+ or K^+,

respectively. These curves were fitted to Boltzmann equation and the voltage for half-maximum activation ($V_{1/2}$) and gating valence (z) were determined (details are given in Figure legends).

Current responses to capsaicin, AITC, menthol, and menthol with AMTB were measured using a continuous protocol at -60 mV. These compounds were applied diluted in external solution using a continuous perfusion system (10 $mL \cdot min^{-1}$). For paclitaxel experiments, to activate TRPV1 and TRPA1, four 1 µM capsaicin pulses of 15 s duration followed by a 60 s pulse of 100 µM AITC were applied, respectively. TRPM8 currents were elicited by a 20 s pulse of 100 µM menthol in different experiments. Then, TRPM8 channel blocker AMTB was used at 10 µM with menthol for 20 s. For oxaliplatin experiments, TRPA1 was activated with a 60 s pulse of 100 µM AITC at the beginning of the protocol followed by activation of TRPM8 as indicated previously after a 2 min wash. In different experiments, TRPV1 was activated with four 1 µM capsaicin pulses of 15 s duration separated by a 1 min wash. Data were acquired at 20 kHz for all the protocols except for the continuous voltage protocols performed to study capsaicin, AITC and menthol responses that were sampled at 1 kHz. These currents were additionally filtered to 2 Hz for plotting. Recordings were performed with an EPC10 amplifier controlled by Patchmaster software (HEKA Elektronik).

To study separately peptidergic and non-peptidergic DRG neurons, before each measurement, coverslips were incubated for 10 min with the fluorescent dye Isolectin- GS-IB4 Alexa Fluor® 568 conjugate (10 $\mu g \cdot mL^{-1}$; Invitrogen) diluted in external solution, followed by two washes. As previously described, IB4 labelling did not alter viability neither electrophysiological parameters of the neurons (Stucky & Lewin, 1999). Cells were visualized with a fluorescent microscope (Axiovert 200 Inverted Microscope, Carl Zeiss) with an excitation filter ET545 and an emission filter ET605 (CHR-49004, Laser 2000 SAD). Cells that did not show fluorescence were considered as IB4(-). Only one cell per dish was recorded and analyzed.

Microelectrode arrays (MEA) recordings.

Extracellular recordings were performed on 60ThinMEAs with 60 electrodes (59 electrodes and one reference electrode) of 30 µm diameter and 200 µm interelectrode distances. Before cell seeding, MEA plates were sterilized for 1 h with UV light and coated during 2 h with poly-L-lysine hydrobromide (50 $\mu g \cdot mL^{-1}$; Sigma-Aldrich). After four washes with deionized water, plates were air-dried in a class II laminar flood hood. One hour before cell seeding, a drop of laminin diluted on DMEM medium (20 $\mu g \cdot mL^{-1}$; Sigma-Aldrich) was added to the center of each plate. Afterwards, the laminin solution was replaced by the cell suspension. Electrical activity was recorded by the MEA 1060 System (Multi Channel Systems MCS GmbH) using the MC_RACK software version 4.3.0 at a sampling rate of 25 kHz.

During the recording, cells were kept in the external solution indicated for patch clamp experiments with a 2 $mL \cdot min^{-1}$ continuous perfusion. TRPV1, TRPA1 and TRPM8 mediated activity was studied with a 1 µM capsaicin application during 20 s followed by 2 min wash, then 100 µM AITC was added during 1 min followed by a 2 min wash, and one application of 100 µM menthol for 20 s. All recordings were performed at 34.5 °C. For analysis, signals were processed with a Butterworth band-pass filter with a high-pass cutoff of 200 Hz. The voltage threshold for spike detection was set at 4.7 times the standard deviation of the noise on each channel using MC_RACK spike sorter. Mean spike frequency data were extracted with the MC_RACK software.

Quantitative RT-PCR.

Total RNA was isolated using the E.Z.N.A.® microElute total RNA kit (Omega Bio-tek). Extracellular RNA samples quantity and purity were analyzed using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific). Possible contaminating DNA was digested using DNase I (Sigma). Then, the RNA extracted was reverse transcribed using the First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Primers targeting rat selective channels were designed and their sequences were: TRPV1: Fw: 5' TGGACGAGGTAAACTGGACT, Rv: AGTTTCTCCCTGAAACTCGG; TRPA1: Fw: 5' AGTGGCAATGTGGAGCGATA Rv: 5' TCCCGTCGATCTCAGCAATG; TRPM8: Fw: 5' GCTACGGACCAGCATTTCAT, Rv: 5' GCTTGTCAATGGGCTTCTT; $Na_V1.7$: Fw: 5' TGGCGTCGTGTCGCTTGT, Rv: 5' TGGCCCTTTGCCTGAGAT; $Na_V1.8$: Fw: 5' TCCTCTCACTGTTCCGCCTCAT, Rv: 5' TTGCCTGGCTCTGCTCTTCATAC; $Na_V1.9$: Fw: 5' ATACGGTGCCCTGATCCTCT, Rv: GGAAGTGAAGGGGCGGAAAT; Rpl29: Fw: 5' ACAGAAATGGCATCAAGAAACCC, Rv: 5' TCTTGTTGTGCTTCTTGGCAAA; HCN1: Fw: 5' CCAACGTCGCGCATGAG, Rv: 5' CAGGGTGAAAGGATGGCTGA; HCN2: Fw: 5' CCACTGCGTGAGGAGATTGT, Rv: 5' ATGGTCCCCTCTCGGATGAT. The complementary DNA (cDNA) was added to the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific) with the forward and reverse primers described and nuclease-free water. To verify the results, we used no template negative controls and reverse transcriptase minus (RT-) negative controls. Amplification and quantification of the cDNA was carried out with the QuantStudio3 Real-Time PCR Instrument (Applied Biosystems) using the following thermal cycling conditions: 50 °C for 2 min, 90 °C for 10 min; 40 cycles of 95 °C during 15 s and 60 °C for 1 min; and final steps of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The expression level of the mRNA was normalized to the housekeeping Rpl29 mRNA levels and calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting.

All western blotting and immunofluorescence procedures conform with BJP guidelines (Alexander et al., 2018). At DIV 8, cell cultures were homogenized, dissolved in RIPA lysis buffer (50 mM Hepes, 140 mM NaCl, 10 % glycerol, 1 % v/v Triton X-100, 1 mM EDTA, 2 mM EGTA, 0.5 % deoxycholate, pH 7.4) with EDTA-free protease inhibitor cocktail (cOmplete™, Sigma-Aldrich) and centrifuged at 14000 rpm for 15 min. The supernatant was collected, and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Cellular protein extracts were mixed (1:1) with 2x sample buffer (0.125 mM Tris-HCl pH 6.8, 40 $mg \cdot mL^{-1}$ SDS, 2 $mg \cdot mL^{-1}$ bromophenol blue, 20 % glycerol and 0.1 M Dithiothreitol) and boiled for 10 min. Thereafter, proteins were electrophoresed on a 7.5 % SDS-PAGE gel and transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad) for 90 min at 100 V using an electrophoretic transfer system (Bio-Rad). Membranes were blocked with non-fat milk at 5 % in TBST (200 mM glycine, 25 mM Tris base, 20 % methanol and 0.05 % Tween, pH 8.3) at room temperature for 1 h for all primary antibodies, except for TRPM8 that required 3 h blocking, according to manufacturer´s instructions. Next, membranes were incubated at 4 °C overnight with the following primary antibodies diluted in 1 % BSA in TBST: rabbit anti-Actin, 1:1000 (Sigma-Aldrich, Cat# A2066, RRID:AB_476693); rabbit anti-TRPV1, 1:1000 (Alomone Labs, Cat# ACC-029, RRID:AB_2040258); rabbit anti-TRPM8, 1:500 (Alomone Labs, Cat# ACC-049, RRID:AB_2040254); rabbit anti-$Na_V1.7$, 1:2500 (Alomone Labs, Cat# ASC-008, RRID:AB_2040198); and rabbit anti-$Na_V1.8$, 1:1000 (Alomone Labs, Cat# ASC-016, RRID:AB_2040188), respectively. After washing the membranes with TBST, they were incubated with the secondary antibody anti-Rabbit IgG, 1:20000 (Sigma-Aldrich, Cat# A0545, RRID:AB_257896). To minimize the number of animals used, blots were cut in three different sections to test the same blot with multiple antibodies. Signals were detected with the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific), visualized in a ChemiDoc™ MP Imaging System (Bio-Rad) and quantified using Image Lab software (BioRad). Protein signals were normalized to actin levels within the same blot.

Materials.

Paclitaxel was dissolved in DMSO to create a stock of 25 mM (paclitaxel stocks stored at -20 °C were used within one month). Before cell treatment, it was diluted in DMEM 10 % FBS 1 % P/S and passed through a 0.2 µm filter. The required quantity was added to the cell medium to have a final concentration of 1 µM. Controls were prepared following the same procedure using only DMSO (0.04 %).

Oxaliplatin was dissolved in ddH_2O to a concentration of 5 $mg \cdot mL^{-1}$. This preparation was vortexed and heated at 60 °C for 5 min to facilitate oxaliplatin dilution, as indicated in manufacturers' instructions. This dilution was passed through a 0.2 µm filter and further diluted in ddH_2O to have a concentration of 0.5 $mg \cdot mL^{-1}$. From this dilution, the required quantity was added to the cell medium to have a final concentration of 1 $mg \cdot mL^{-1}$. Controls were prepared following the same procedure but adding filtered ddH_2O to the cell medium.

For electrophysiological experiments, capsaicin, AITC, menthol, AMTB and PF04885614 were dissolved in DMSO to have stock concentrations of 10 mM, 1 M, 1 M, 100 mM and 100 µM, respectively. 4,9-anhydro-TTX and ProTx II stock solution were prepared in water to a concentration of 500 µM and 50 µM, respectively. The day of the experiment, these solutions were diluted in the external solution to reach the final concentration indicated for each experiment. Final concentration of DMSO was 0.01 % of the total volume for capsaicin, AITC, menthol and AMTB, and 0.075 % for PF04885614.

Data and Statistical analysis.

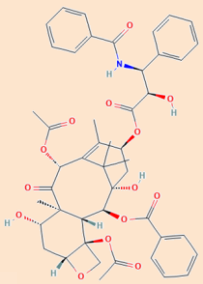
Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018), with the exceptions that: data collection and analysis were not blinded because they were performed by the same researcher and, because effects and standard deviation could not be previously predicted, data were evaluated through experiments to minimize the number of animals used. Data were statistically analyzed using the GraphPad Prism® 9.0.0 software (GraphPad, San Diego, CA, USA). For quantitative variables, first we assessed whether data followed a gaussian distribution using D'Agostino-Pearson (omnibus k2) normality test. Data with a normal distribution are expressed as mean ± SD and analyzed with unpaired t-tests as indicated. As the heterogeneity of DRG neurons is high, and our sample size was not sufficient to determine multiple associations, we restricted our statistical analysis to the defined groups (treatment and sex type) that were analyzed using the Two-way ANOVA test for parametric data, with the Tukey's post-hoc test when F achieved statistical significance or Kruskal-Wallis with Dunn's multiple comparisons test for non-parametric data.

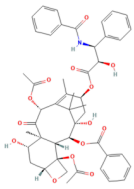
Non-normal distribution data are expressed as median (Q25-Q75) and analyzed with Mann-Whitney test. For qualitative variables, data are expressed as percentage and analyzed with Fisher's exact test. Significant differences were set to $P < 0.05$. For patch clamp experiments, *n* represents the number of cells measured and corresponds to the number of independent experiments, as a single neuron was monitored in each crystal; *N* denotes the number of rats used. For immunofluorescence

measurements, *n* represents the number of neurons and *N* the number of independent experiments; and, for MEA, *n* denotes the number of active electrodes. Statistical analysis was based on the number of independent experiments. Details of the statistics are reported in the Figure legends.

Results

Paclitaxel model





Paclitaxel model

Results obtained from Publication 1

The results of the paclitaxel-induced neuropathy *in vitro* model were published in the British Journal of Pharmacology:

Publication 1.

- Villalba-Riquelme, E., de la Torre-Martínez, R., Fernández-Carvajal, A., & Ferrer-Montiel, A. (2022). Paclitaxel in vitro reversibly sensitizes the excitability of IB4(−) and IB4(+) sensory neurons from male and female rats. *British Journal of Pharmacology*, 179(14), 3693– 3710. https://doi.org/10.1111/bph.15809

Paclitaxel reversibly sensitized small DRG neurons

To investigate the direct effect of paclitaxel on the peripheral nervous system, we developed a long-term DRG neuronal culture that could survive up to 10 days *in vitro*. Before treatment, we incubated the cells for 5 days (until DIV 5) to allow the sprouting of the axons and reestablishment of transport mediated mechanisms. At that point, we treated them during 24 h with 1 µM paclitaxel or its vehicle (0.04 % DMSO) (from DIV 5 to DIV 6). Then, these agents were removed changing the cell medium with fresh medium. The time-course of paclitaxel evoked sensitization and desensitization was investigated analyzing the electrical activity of small DRG neurons at DIV 6 (0 h after eliminating the treatment (post-treatment)), DIV 8 (48 h post-treatment), and DIV 10 (96 h post-treatment) (Figure 10).

As a result, we observed that paclitaxel significantly increased the excitability of small DRG neurons. This effect peaked 48 h after exposure, where we could observe increased spontaneous activity, tonic firing and rheobase in comparison to its vehicle. At 96 h post-treatment, these differences dissipated, detecting only a statistically significant increase in the tonic firing. These data suggested a reversible effect of paclitaxel treatment (Figure 10).

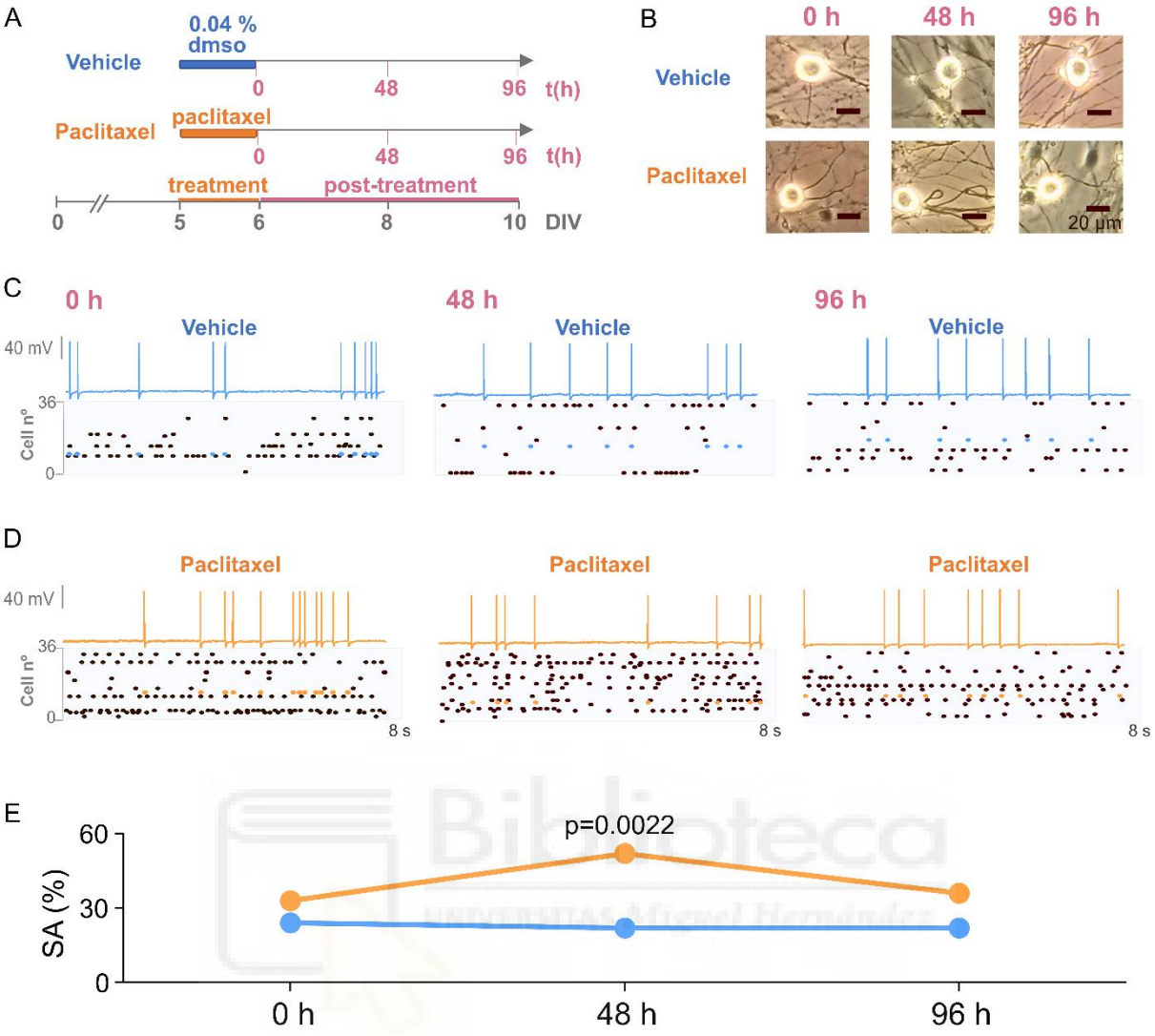


Figure 10. Paclitaxel increased the electrical activity of small DRG neurons 48 h after treatment. **A)** Schematic representation of the experimental design used for investigating paclitaxel effect on DRG neurons. **B)** Representative images of the DRG neurons throughout the culture 0 h, 48 h and 96 h after paclitaxel or vehicle exposure. **C, D)** Representative registers (top) and raster plots (down) of the spontaneous activity at 0 h, 48 h, and 96 h after vehicle (**C**) or paclitaxel (**D**) treatment. **E)** Percentage of small DRG neurons with spontaneous activity at 0 h, 48 h, and 96 h after paclitaxel or vehicle treatment. Fisher's exact test. $P < 0.05$ values are shown. Figure extracted from this publication (Villalba-Riquelme et al., 2022).

Paclitaxel increased the electrical activity in IB4(-) and IB4(+) neurons

Since DRG cultures are very heterogeneous because they are constituted by different neuronal populations, we investigated the effect on the two major neuronal types, IB4(-) and IB4(+), of small DRG neurons, as indicated in the Materials and Methods section. As a result, paclitaxel similarly reversibly increased the excitability of both IB4(-) and IB4(+) neurons, peaking 48 h post-treatment (Tables S2 and S3, Publication 1). At this time point, IB4(-) showed significantly increased spontaneous activity (SA) (from 31 to 67 %), that could be influenced by the depolarization observed in their resting membrane potential (RMP) (Figure 2, Publication 1). SA was also significantly higher in IB4(+) neurons treated with paclitaxel (from 0 to 33 %). In this case, a tendency towards a more depolarized RMP could be noticed, although not significant differences were observed (Figure 2, Publication 1). Active membrane properties such as the tonic action potential (AP) firing, and its firing frequency were also significantly higher in both populations (Figure 11). The current needed for evoking these action potentials was also considerably reduced by paclitaxel 48 h post-treatment (Figure 11). Since the major differences were observed 48 h after removing the treatment, we focused on this time point for the following experiments.

These results suggested that paclitaxel could be altering the action potential parameters on the neurons. After a detailed analysis of these parameters, we could observe that paclitaxel significantly depolarized the afterhyperpolarization phase of both neuronal populations (Figure 4, Publication 1), thus facilitating the repetitive firing previously observed (Figure 11). In IB4(-) neurons, paclitaxel also promoted a faster repolarization time and recovery from the hyperpolarization phase. Other parameters such as the time to peak, voltage threshold, overshoot, amplitude, maximum upstroke slope did not differ between the paclitaxel and vehicle treated groups (Figure 4, Publication 1).

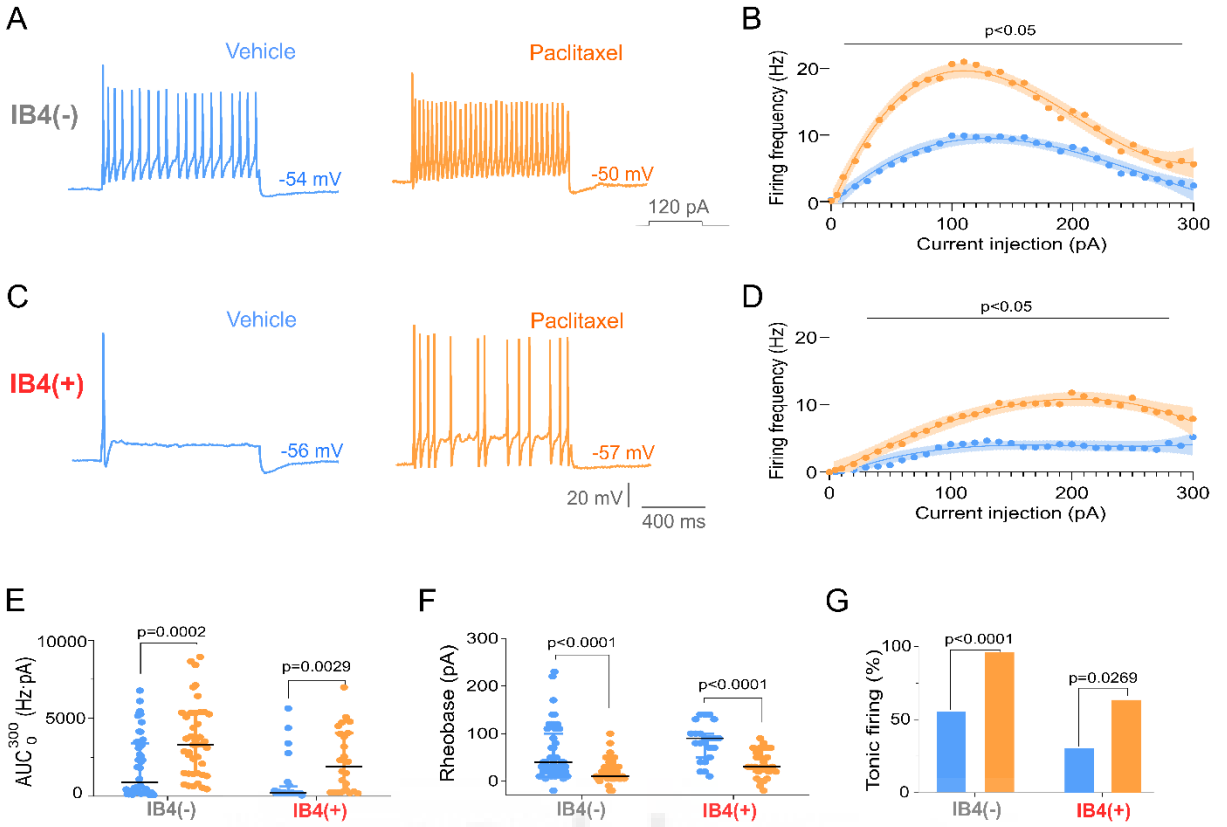


Figure 11. Paclitaxel potentiated electrically evoked AP firing of IB4(-) and IB4(+) small-DRG neurons. (A and C) Representative recordings of AP firing evoked by a 1 s of current injection pulse of 120 pA for IB4(-) **(A)** and IB4(+) **(C)** neurons exposed to vehicle (blue traces) or paclitaxel (orange traces) recorded 48 h post-treatment. **(B and D)** Firing frequency (nº of APs evoked during 1 s depolarizing pulse) at each current injected from 0 to 300 pA in 10 pA intervals for IB4(-) **(B)** and IB4(+) **(D)** neurons exposed to vehicle or paclitaxel. **(E)** Area under the curve (AUC_0^{300}) obtained plotting the firing frequency as a function of the injected current in the range of 0 to 300 pA. **(F)** Rheobase values for IB4(-) and IB4(+) small DRG neurons exposed to vehicle or paclitaxel. **(G)** Percentage of neurons exposed to vehicle and paclitaxel exhibiting tonic firing. Data from vehicle treated neurons are colored in blue and paclitaxel treated in orange. Data are expressed as Median (IQR). Each dot represents the values measured for each cell recorded. Data were collected 48 h post-treatment. *N* = 20, for IB4(-) with *n* =49 for vehicle and *n* = 42 for paclitaxel; *N* = 13, for IB4(+) with *n* = 25. Statistical analysis was performed using the Mann-Whitney test (B, D, E, F) or the Fisher's exact test (G). *P* < 0.05 values indicating parameters significantly different from vehicle are shown. Figure extracted from this publication (Villalba-Riquelme et al., 2022).

Paclitaxel increased Na_V channel currents in IB4(-) and IB4(+) neurons

To elucidate the molecular mechanisms that could be explaining the increased excitability of the neurons, we investigated the activity of Na_V channels at the peak of the activity (48 h post-treatment). As a result, paclitaxel treatment hyperpolarized the $V_{1/2}$ of the Na_V current of IB4(-) neurons. Meanwhile, on the IB4(+) group, it promoted a faster recovery from inactivation (Figure 5, Publication 1). Then, we used specific blockers of the $Na_V1.7$ (ProTx II) and $Na_V1.8$ (PF04885614) subunits to investigate its specific contribution to these currents. To obtain the $Na_V1.7$ and $Na_V1.8$ equivalent currents, we subtracted the non-blocked current to the total Na_V current. Application of ProTx II did not seem to differentially affect the Na_V currents. However, the $Na_V1.8$ current seemed to be higher in IB4(+) neurons after paclitaxel treatment, with an apparent effect on hyperpolarized potentials on IB4(-) (Figure 6, Publication 1). To further investigate this effect, we performed a voltage protocol previously designed for isolating $Na_V1.8$ current (Soriano et al., 2019). When we measured the current density at different potentials, we could observe a major increase in the $Na_V1.8$ current density of IB4(+) neurons at different potentials and a modest increase in IB4(-) neurons at -40 mV. $Na_V1.8$ currents of IB4(+) also exhibited a strong 9 mV hyperpolarizing shift of the $V_{1/2}$. To find a potential explanation of these changes, we evaluated the $Na_V1.7$ and $Na_V1.8$ mRNA and protein levels using qPCR and Western blot, respectively (Figure S3 and Table S5, Publication 1). As a result, we could not observe any upregulation in the mRNA or protein quantity of these channels. However, increased $Na_V1.8$ immunoreactivity was detected in immunocytochemistry experiments, suggesting a possible increase of the channel in a subset of neurons (Figure S4, Publication 1).

Paclitaxel affected A-type K^+ currents in IB4(-) and IB4(+) DRG neurons

Due to the relevance of K_V channels in controlling the membrane excitability, we analyzed the effect of paclitaxel on the fast-inactivating (K_A) and delayed-rectifying (K_{DR}) potassium currents. There, a major effect of paclitaxel in K_A current could be observed (Figure 7, Publication 1). In IB4(+) neurons, K_A current density was reduced at depolarizing voltages near the voltage threshold of the cells. IB4(-), on the contrary, were characterized by higher K_A currents at very positive potentials ≥ 10 mV. Both types of neurons also exhibited a shift to more depolarizing potentials in the conductance-voltage relationship 48 h after paclitaxel treatment. Thus, paclitaxel promoted a high threshold K_A current in IB4(-) neurons while inhibited a low threshold K_{DR} current in IB4(+) neurons. Paclitaxel effect on K_{DR} currents was much less apparent, only showing a shift to more positive potentials and a lower current density at -40 mV (Figure 7, Publication 1).

Paclitaxel sensitized IB4(-) and IB4(+) neurons from both male and female rats

The increasing evidence of sex differences on pain signaling, incited as to investigate if paclitaxel sensitization could affect in a different way male and female neurons. To do this, we analyzed separately the paclitaxel effect on the DRG neurons extracted from male and female rats 48 h post-treatment. Studying the rheobase, firing frequency and RMP of these neurons, male and female neurons showed a similar trend to hyperexcitability, but it reached statistical significance only in female neurons, suggesting a more prominent effect of paclitaxel on this sex (Figure 8, Publication 1).

Paclitaxel increased TRPV1 and TRPM8 channel functionality

As key thermal and noxious sensors, TRPV1, TRPA1 and TRPM8 are thought to play a pivotal role in the development of CIPN (Cabañero et al., 2022). First, we analyzed its activity after application of its agonists using microelectrode arrays (MEA). Paclitaxel significantly augmented the action potential firing after stimulation of capsaicin and menthol but not with AITC, suggesting upregulation of the TRPV1 and TRPM8 ion channels but not of TRPA1. Analyzing the mRNA expression of these channels did not show any transcriptional alteration of TRPV1, TRPA1 or TRPM8 (Table S5, Publication 1). However, we could detect a significant increase in TRPV1 protein levels using Western blot (Figure S3, Publication 1). This TRPV1 rise was also detected with immunocytochemistry measurements, where we could also observe increased immunoreactivity for the TRPM8 channel without altering TRPA1 (Figure 9, Publication 1). A detailed analysis of the activity of TRPV1, TRPA1 and TRPM8 after paclitaxel treatment was performed with the patch clamp technique. In agreement with the previous results, paclitaxel significantly increased the current density in response to capsaicin and menthol without altering the response to AITC. This effect was found only in the IB4(-) subtype although IB4(+) showed a tendency to higher TRPV1 activity (Figure 12).

Comparison between male and female DRG neurons did not show any sex differences in TRPV1 response. However, menthol evoked currents were significantly higher in male treated neurons than in their female counterparts (Figure 12). Thus, our results indicated the presence of sexual dimorphism in the alterations produced by paclitaxel on the TRPM8 activity.

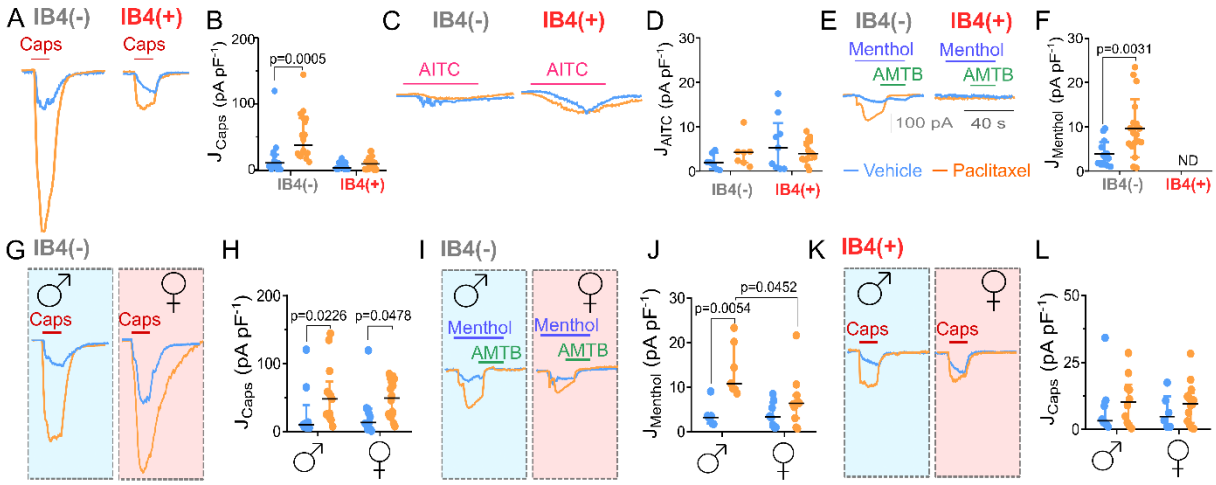
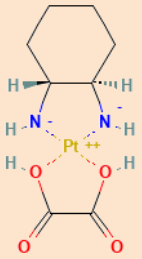
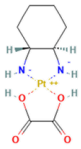


Figure 12. Paclitaxel increases TRPV1 and TRPM8 activity in IB4(-) neurons. A, C, E) Representative inward currents in response to 1 µM capsaicin (**A**), 100 µM AITC (**C**), and 100 µM menthol (**E**). **B, D, F)** Peak current density registered in response to capsaicin (**B**), AITC (**D**), and menthol (**F**). Mann-Whitney test for capsaicin and Unpaired t-test for menthol responses. **G, I, K)** Representative current responses for to capsaicin (**G**) and menthol (**I**) for IB4(-) and to capsaicin (**K**) for IB4(+) male and female neurons. **H, J, L)** Peak current densities of capsaicin IB4(-) responses (**H**), menthol IB4(-) responses (**J**), and capsaicin IB4(+) responses (**L**) of male and female neurons. Kruskal-Wallis with Dunn's multiple comparisons test. Statistically significant $p < 0.05$ values are indicated in each graph. Recordings performed 48 h post-treatment. Figure extracted from this publication (Villalba-Riquelme et al., 2022).

In conclusion, with this study we could develop a preclinical *in vitro* model that reproduced and extended the results provided by animal models. This model allowed us to observe and analyze the direct and reversible effect of paclitaxel on the DRG cell cultures. Our results suggest that TRPV1, TRPM8 and $Na_V1.8$ could be crucial in the alteration of nociceptors excitability and the development of pain after paclitaxel administration. Furthermore, we could detect a sexual dimorphism in paclitaxel response with female rat nociceptors being more sensitive to this effect. Thus, our *in vitro* preclinical model provides a powerful tool for investigating the molecular mechanisms underlying peripheral neuropathies and for testing potential therapeutic drugs that could control the pain symptoms.

Oxaliplatin model





Oxaliplatin model

Results under preparation for Publication

Oxaliplatin dependent effect was investigated using the protocol described in Figure 13A. Briefly, oxaliplatin concentration was set to 1 $\mu g \cdot mL^{-1}$, a clinically relevant level found in patients (Ehrsson et al., 2002; Kern et al., 1999). Then, two different conditions were tested: incubation during 24 h between DIV 5 and DIV 6 in culture, and incubation during 48 h between DIV 4 and DIV 6. Vehicle group was exposed to the oxaliplatin vehicle (distilled water) instead of oxaliplatin. To analyze the time-dependent effect of oxaliplatin treatment, the spontaneous activity (SA), tonic firing and rheobase were measured on small DRG neurons 0 h, 48 h, and 96 h after removing the agent.

Oxaliplatin reversibly sensitized the activity of small DRG neurons in a time-dependent manner

As depicted in Figure 13B-C, oxaliplatin alterations were time-dependent with higher incubation time (48 h) leading to higher excitability than shorter exposures (24 h). Indeed, after 24 h exposure of oxaliplatin, the percentage of neurons with tonic firing (Tonic%) remained very close to the measurements obtained in the vehicle group during all the culture (~46 %). Only a small tendency to increase the Tonic% to 52 % was observed at 48 h after removing oxaliplatin (Figure 13C).

A more prominent effect was observed with longer oxaliplatin incubations. After 48 h exposure, oxaliplatin significantly increased the percentage of neurons with SA from 13 to 40 % (48 h post-treatment, Table 3). A tendency to augment the SA was also apparent during all the culture, as shown at 0 and 96 h after removing the agent (Table 3). Complementarily, the tonic firing increment peaked 0 h after exposure changing from 47 % after vehicle exposure to 79 % with oxaliplatin (Figure 13C, Table 3). The enhanced excitability was also detected in the rheobase of the cells. Indeed, just after treatment, the rheobase was reduced from a median of 70 pA with vehicle to 30 pA at 0 h after oxaliplatin treatment (Table 3). Since the major sensitizing effect was observed after the 48 h exposure and most of the bibliography also used a similar time of exposure as a clinically relevant incubation time, for further characterization we focused on this exposure time (Anand et al., 2010; Riva et al., 2018).

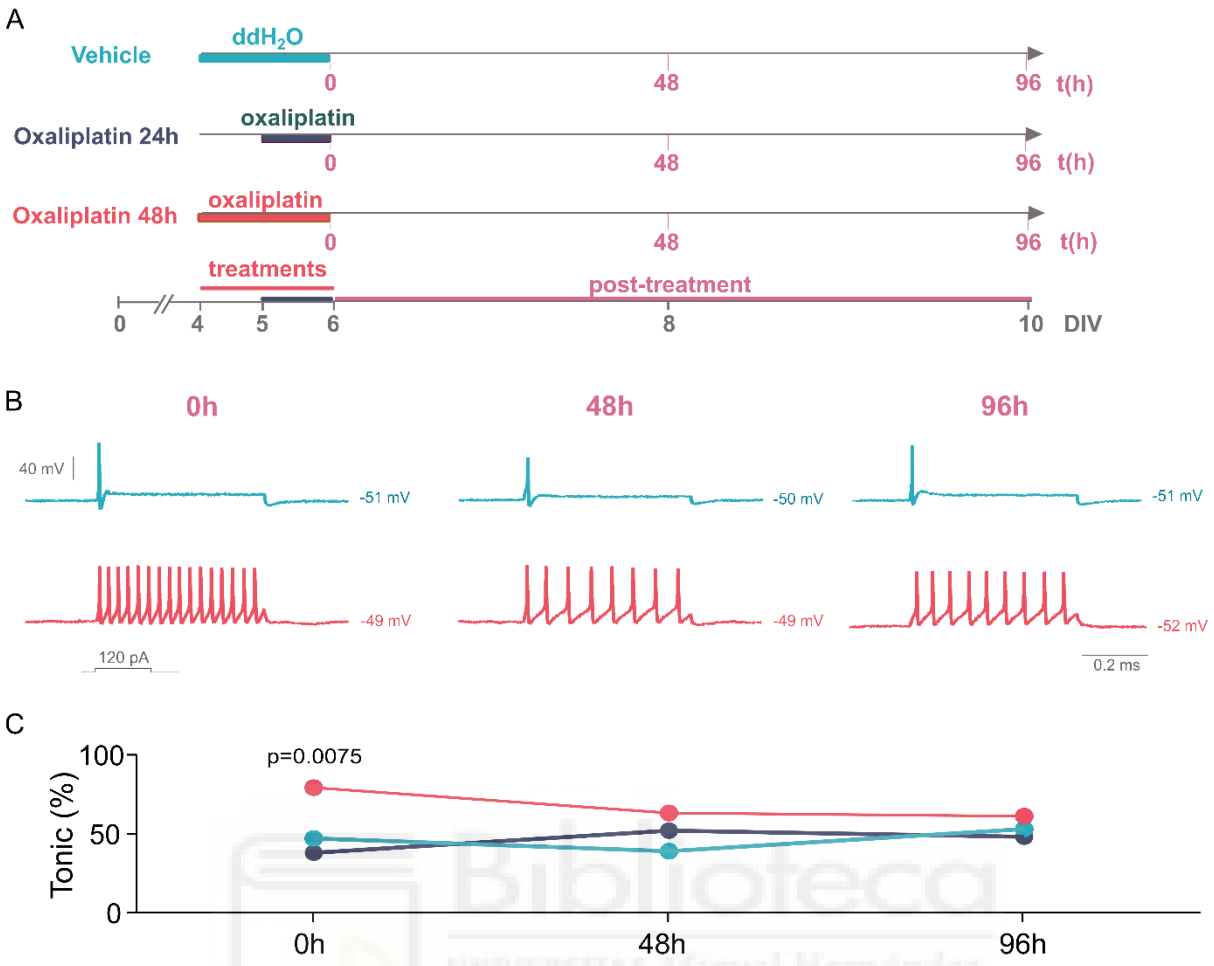


Figure 13. Time dependent effect of oxaliplatin on the electrogenic activity of sensory neurons. A) Diagram of the time course of the experiments performed for characterizing DRG neurons activity after oxaliplatin exposure. Cells were incubated with the vehicle (ddH_2O, double distilled water) for the control group or with 1 $\mu g \cdot mL^{-1}$ oxaliplatin during 24 h between days 5 and 6 (5-6 DIV), or 48 h between days 4 and 6 of culture (4-6 DIV), respectively. Registers were performed at 0 h (DIV 6), 48 (DIV 8) and 96 (DIV 10) post-treatment. **B)** Representative registers of the most common firing pattern found when a 1s depolarizing pulse of 120 pA was applied at 0 h, 48 h, and 96 h post-treatment. **C)** The percentage of neurons with tonic firing (Tonic) at 0 h (DIV 6), 48 (DIV 8) and 96 (DIV 10) post-treatment are depicted. Vehicle and oxaliplatin groups were compared using the Fisher's exact test. *P* values for data significantly different from vehicle are indicated.

Table 3. Time course of oxaliplatin effect on electrogenic activity of sensory neurons.

	0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin
SA (%) n_{SA}/n_{total}	22 8/36	39 15/38	13 5/38	40* 12/30 p=0.0223	21 8/38	42 15/36
Rheobase (pA)	70 (20-130)	30 (13-60)* p=0.0004	45 (20-103)	40 (25-70)	45 (30-113)	50 (20-100)
Tonic (%) n_{tonic}/n_{total}	47 17/36	79* 30/38 p=0.0075	39 15/38	63 19/30	53 20/38	61 22/36

The percentage of neurons with spontaneous activity (SA), rheobase and percentage of neurons with tonic firing (Tonic) at 0 h (DIV 6), 48 h (DIV 8) and 96 h (DIV 10) post-treatment are depicted. Vehicle and oxaliplatin were compared using the Mann-Whitney test for rheobase, and the Fisher's exact test for SA and Tonic firing. *N* = 15, *n* = 36 for vehicle and *n* = 38 for oxaliplatin 0 h after treatment; *n* = 38 for vehicle and *n* = 30 for oxaliplatin, 48 h after treatment; *n* = 38, for vehicle, n=36 for oxaliplatin, 96 h after treatment. *$P < 0.05$ significantly different from vehicle, and values highlighted in red.

As it is possible to appreciate, the increase in the electrical activity parameters peaked immediately after exposure except for spontaneous activity where similar values were obtained 0 h, 48 h and 96 h after removing the agent (Table 3). Thus, for the following experiments, we focused on the analysis of the functional and molecular changes evoked 0 h post-treatment.

Oxaliplatin predominantly increased the excitability of IB4(+) small DRG neurons

To determine which cell types could be altered by oxaliplatin, the two major populations of nociceptors, IB4(-) and IB4(+), were analyzed separately. IB4(-) did not show a significant difference in the resting membrane potential (RMP), rheobase or percentage of neurons with tonic firing after oxaliplatin exposure at any time measured (Table 4). However, oxaliplatin seemed to produce a small increase in the percentage of neurons with SA at DIV8 from 18 to 58 %, and at DIV10, from 26 to 62 %.

Table 4. Electrical activity of IB4(-) neurons after vehicle or oxaliplatin exposure.

	IB4 (-)					
	0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin
n	20	21	22	19	23	21
RMP (mV)	-54 ± 8	-51 ± 6	-50 ± 6	-50 ± 7	-52 ± 6	-52 ± 6
C (pF)	16 (13-21)	13 (11-22)	15 (13-22)	17 (14-19)	19 (15-25)	15 (13-20)
SA (%)	35 n=7/20	43 n=9/21	18 n=4/22	58 n=11/19* p=0.0115	26 n=6/23	62 n=13/21* p=0.0319
SA f (Hz)	2 ± 2	2 ± 1	1 ± 1	2 ± 2	2 ± 2	1 ± 1
Rheobase (pA)	35 (10-95)	20 (5-65)	30 (18-75)	30 (10-50)	30 (20-110)	30 (10-65)
Tonic (%)	55 n=11/20	67 n=14/21	45 n=10/22	63 n=12/19	57 n=13/23	62 n=13/21

Table indicating the number of recordings for each group (*n*), resting membrane potential (RMP), capacitance (C), percentage of neurons with spontaneous activity (SA), spontaneous activity frequency (SA f), rheobase, and percentage of neurons with tonic firing (Tonic) of IB4(-) exposed to vehicle or oxaliplatin for 48 h exposure. Statistical analysis performed between vehicle and oxaliplatin groups within each subtype were: Unpaired t-test for RMP, SA f and R_{input}; Mann-Whitney test for capacitance and rheobase; and Fisher's exact test for SA and tonic firing. Significant differences from vehicle (*****$P < 0.05$) are indicated in the table and highlighted in red.

On the other hand, IB4(+) neurons showed an increase in the excitability immediately after oxaliplatin exposure (0 h post-treatment), dissipating through the culturing time (Table 5). There, a statistically significant increase in the percentage of neurons with SA was found (Table 5, Figure 14B). The altered SA % did not seem to be correlated with a more depolarized RMP nor a higher SA f, although a tendency to depolarize the RMP could be observed (53 ± 6 mV to 51 ± 6 mV at 0 h post-treatment, Figure 14C-D). It could also be noted a smaller capacitance in the oxaliplatin treated group (Figure 14E). R_{input} values also showed a tendency to increase in IB4(+) neurons, suggesting that more opened channels could be present in the cell membrane after oxaliplatin treatment (Figure 14F).

Table 5. Electrical activity of IB4(+) neurons after vehicle or oxaliplatin exposure.

	IB4 (+)					
	0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin
n	36	31	17	18	20	24
RMP (mV)	53 ± 6	-51 ± 6	-50 ± 6	-50 ± 8	-50 ± 7	-52 ± 7
C (pF)	25 (21-30)	21 (19-25)* p=0.0325	28 (24-32)	26 (23-30)	26 (21-30)	24 (21-29)
SA (%)	14 n=5/36	39 n=12/31* p=0.0259	6 n=1/17	33 n=6/18	25 n=5/20	38 n=9/24
SA f (Hz)	0.8 (0.1-2.6)	0.7 (0.1-1.1)	1.0 (0.3-2.2)	0.9 (0.3-1.6)	0.1 (0.1-2.3)	0.1 (0.1-0.4)
Rheobase (pA)	80 (50-130)	30 (20-50)* p=0.0001	60 (33-110)	40 (28-73)	45 (30-110)	65 (25-100)
Tonic (%)	39 n=14/36	87 n=27/31 p<0.0001	35 n=6/17	67 n=12/18	55 n=11/20	71 n=17/24

Table similar to Table 4 obtained for IB4(+) neurons. Oxaliplatin and vehicle groups were analyzed using the following statistical analysis: Unpaired t-test for RMP; Mann-Whitney test for capacitance, SA f and rheobase; and Fisher's exact test for SA and Tonic firing. Significant differences from vehicle are indicated in the table with *$P < 0.05$ and highlighted in red.

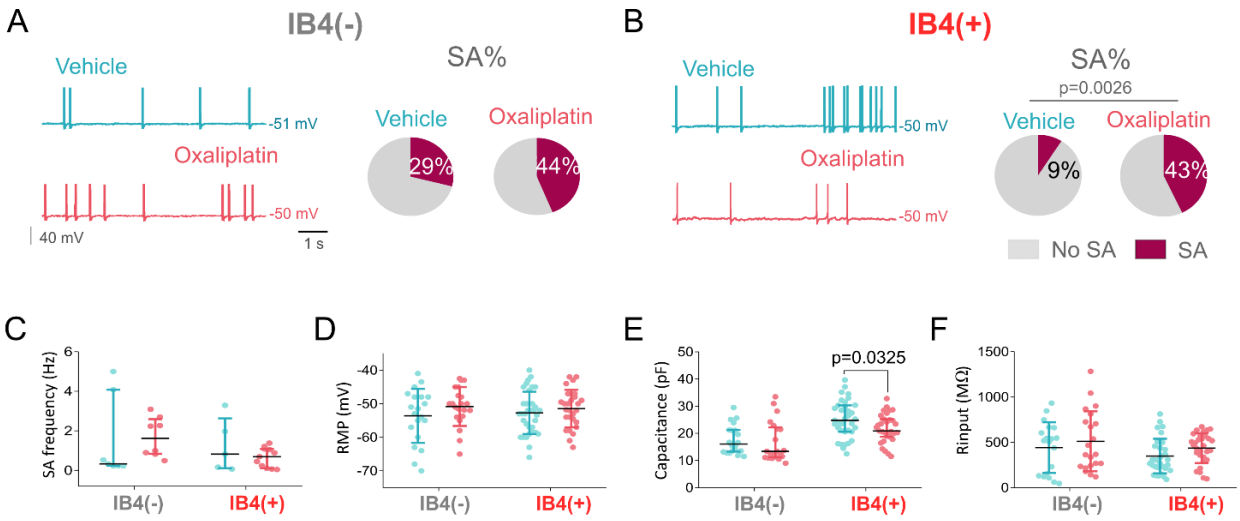


Figure 14. Oxaliplatin increased the number of IB4(+) neurons displaying spontaneous activity. (A and B) Representative AP recordings fired spontaneously (0 pA) of IB4(-) (**A**) and IB4(+) (**B**) neurons 0 h after finishing vehicle (blue) or oxaliplatin (red) 48 h treatment. The percentage of neurons firing SA for each group is indicated on the right as pie charts. The SA (%) is indicated inside the chart. (**C**) Frequency of the spontaneous AP firing in 30 s recordings. (**D**) Resting membrane potential (RMP) for IB4(-) and IB4(+) neurons 0 h post-treatment with vehicle or oxaliplatin. (**E**) Capacitance of the cells recorded in vehicle and oxaliplatin groups for IB4(-) and IB4(+) neurons. (**F**) Input resistance (R_{input}). Statistical analysis was performed using the Fisher´s exact test (**A and B**), the Unpaired t-test (**C, D and F**), or the Mann-Whitney test (**E**). P-values significantly different ($P < 0.05$) from vehicle are indicated.

After applying different current stimulus, the firing frequency of both neuronal populations were also differently affected by oxaliplatin. IB4(-) evoked electrical activity did not significantly change after oxaliplatin exposure (Table 4). Nevertheless, a tendency of a decreased rheobase from 35 (10-85) pA to 20 (5-60) pA and higher firing frequency from 666 (245-3848) Hz·pA to 1660 (755-2325) Hz·pA could be noticed (Table 4, Figure 15E-F).

IB4(+) neurons exhibited a significantly lower rheobase (from 80 (50-130) pA to 30 (20-50) pA), higher firing frequency with a higher area under the curve (from 343 (168-2743) Hz·pA to 2975 (1505-4088) Hz·pA), and bigger percentage of neurons with tonic firing (from 39 % to 87 %) (Table 5, Figure 15). These results could indicate that IB4(+) neurons alterations were responsible of the major change detected in the culture electrogenicity after oxaliplatin application shown in Figure 13.

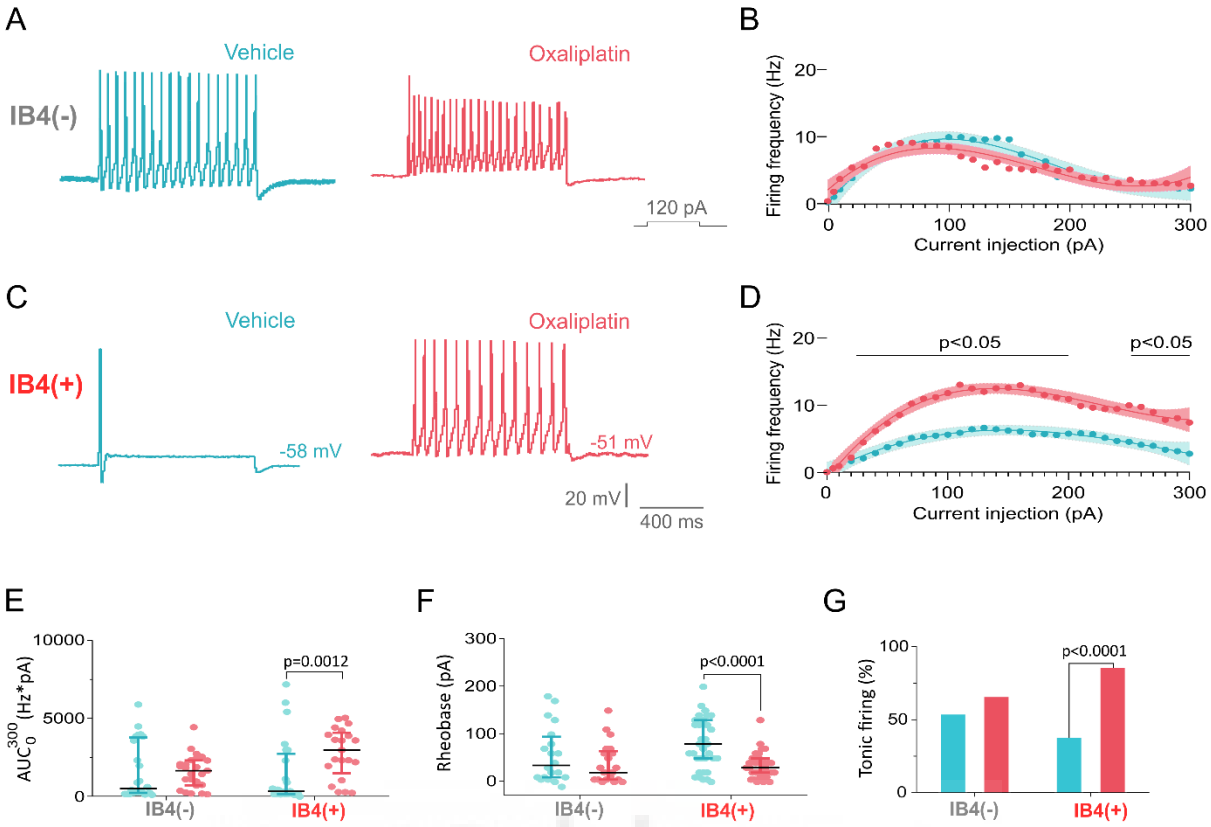


Figure 15. Oxaliplatin potentiated electrically evoked AP firing of IB4(+) small-DRG neurons. A and C) Representative recordings of AP firing evoked by a 1 s-current injection pulse of 120 pA for IB4(-) **(A)** and IB4(+) **(C)** neurons exposed to vehicle (blue traces) or oxaliplatin (red traces) recorded 0 h post-treatment. **B and D)** Firing frequency (nº of APs evoked during the 1 s depolarizing pulse) at each current injected from 0 to 300 pA in 10 pA intervals for IB4(-) **(B)** and IB4(+) **(D)** neurons exposed to vehicle or oxaliplatin. **(E)** Area under the curve (AUC_0^{300}) obtained from plotting the firing frequency as a function of the injected current in the range of 0 to 300 pA. **F)** Rheobase values for IB4(-) and IB4(+) small DRG neurons exposed to vehicle or oxaliplatin. **G)** Percentage of neurons exposed to vehicle and oxaliplatin exhibiting tonic firing. Data from vehicle treated neurons are colored in blue and oxaliplatin treated in red. Each dot represents the values measured for each cell recorded. Lines and error bars indicate the median (IQR) values. Data were collected 0 h post-treatment. *N* = 15, for IB4(-) with *n* = 20 for vehicle, and *n* = 21 for oxaliplatin; *N* = 18, for IB4(+) with *n* = 36 for vehicle, and *n* = 31 for oxaliplatin. Statistical analysis was performed using the Mann-Whitney test **(B, D, E, F)** or the Fisher's exact test **(G)**. $P < 0.05$ values indicating significantly different from vehicle are shown.

Oxaliplatin altered the action potential waveform of IB4(+) neurons

Action potential parameters alterations could give us a hint on potential molecular targets that could be affected by oxaliplatin and could explain its consequent exacerbated excitability. Hence, we dissected different parts of the AP at 0 h after vehicle or oxaliplatin treatment. The outcome of this analysis is shown in Figure 16.

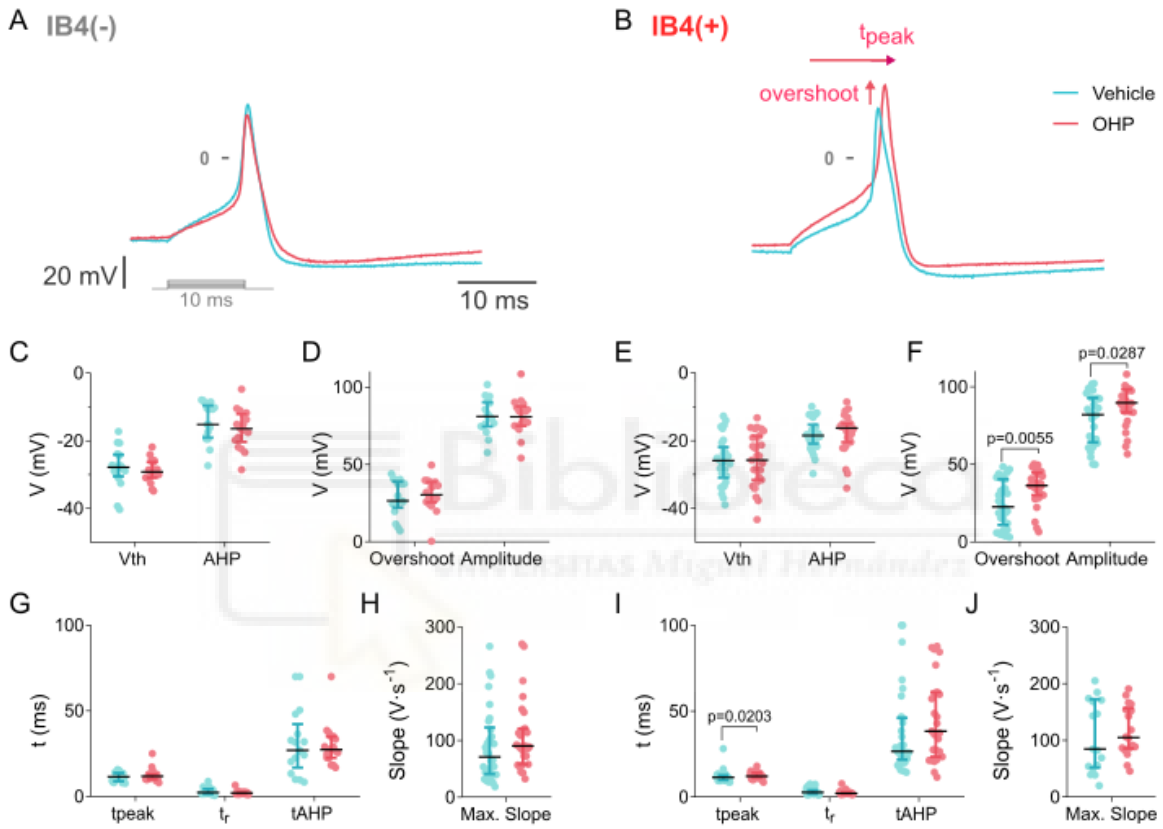


Figure 16. Oxaliplatin altered the action potential parameters of IB4(+) neurons. A, B) Representative APs of IB4(-) (**A**) and IB4(+) (**B**) neurons exposed to vehicle (blue) or oxaliplatin (red). **C, E)** Action potential threshold (V_{th}) and amplitude of the afterhyperpolarization phase (AHP) recorded for IB4(-) (**C**) and IB4(+) (**E**) neurons. Unpaired t-test**. D, F)** Overshoot and Amplitude for IB4(-) (**D**) and IB4(+) (**F**). Unpaired t-test for overshoot, Mann-Whitney test for amplitude. **G, I**) Time to peak (t_{peak}), repolarization time (t_r), and t_{AHP} parameters for IB4(-) (**G**) and IB4(+) (**I**) neurons. Mann-Whitney test. **H, J**) Maximum upstroke slope for IB4(-) (**H**) and IB4(+) (**J**) neurons. Mann-Whitney test. Lines indicate median (IQR) values. Statistically significant ($P < 0.05$) values between vehicle and oxaliplatin groups are indicated in each graph.

As expected, the AP parameters measured were not significantly affected on IB4(-) neurons (Figure 16A, C-D, G-H). For IB4(+) neurons, oxaliplatin affected the upstroke phase of the AP. There, we found a significant increase in the overshoot (from 23 (11-40) mV to 36 (30-45) mV) and amplitude after this treatment (from 79 ± 17 mV to 88 ± 14 mV) (Figure 16B, F). These changes indicate that oxaliplatin could generate a stronger depolarization upon the AP. Oxaliplatin treated IB4(+) cells also needed more time to reach the peak of the action potential (Figure 16I). In Figure 16E and 16I, it is also possible to observe a tendency towards a lower amplitude and duration of the AP hyperpolarization. This effect could also explain the higher AP frequency evoked in treated neurons. Similar data between vehicle and oxaliplatin were obtained for t_r and V_{th} (Figure 16E, I).

Oxaliplatin altered Na_V currents on IB4(+) neurons

To determine the potential molecular mechanisms involved in the increased excitability of IB4(+) neurons after oxaliplatin treatment, we first analyzed the activity of essential determinants of the AP firing, the voltage-gated sodium channels (Na_V). We investigated the total sodium current density in response to different voltage injections from -80 to 40 mV. As depicted in Figure 17A-B, we could not find significant differences in the J-V and G-V curves. These results suggest that the alterations observed in the small DRG neurons electrogenicity are not due to higher Na_V current density nor shift to more negative potentials. However, changes after oxaliplatin exposure became apparent analyzing the kinetics of Na_V currents (Figure 17A). Interestingly, oxaliplatin seemed to increase the duration of Na_V current. IB4(+) neurons had slower inactivation kinetics of the Na_V currents immediately after oxaliplatin treatment producing wider current responses with a decay time constant τ_{fast} = 0.8 (0.4-1.1) ms at 10 mV (Figure 17A, C). In addition, oxaliplatin treated IB4(+) neurons recovered faster from inactivation after short hyperpolarization intervals (10 ms) between pulses than in the vehicle group (Figure 17E), probably contributing to the higher repetitive firing pattern observed after oxaliplatin treatment (Figure 15C).

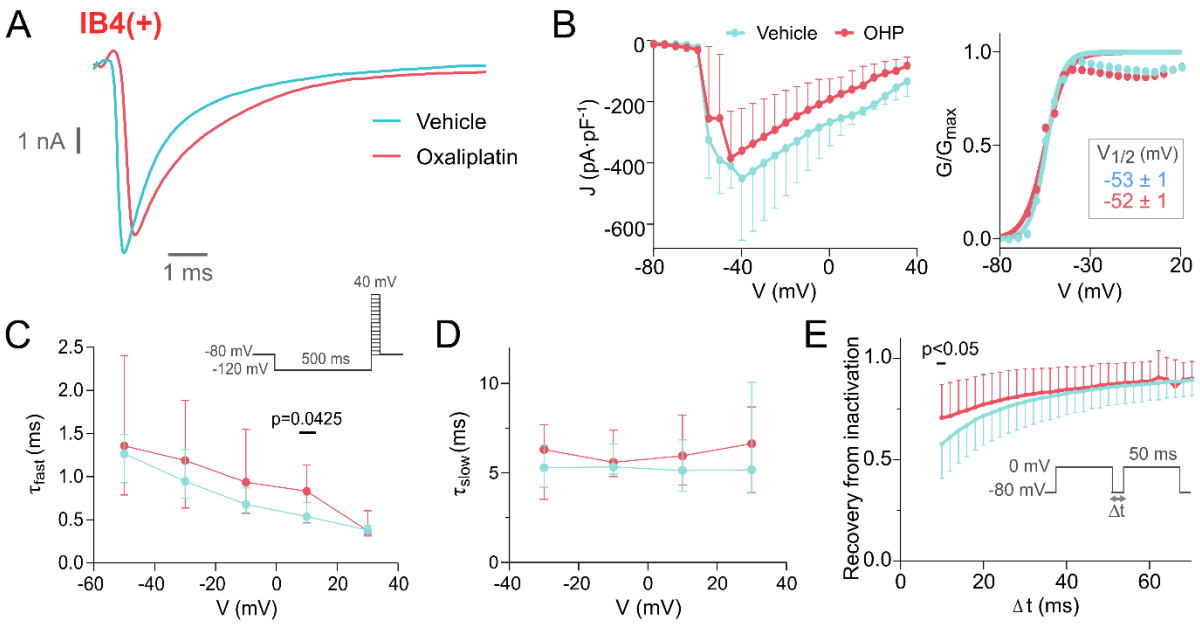


Figure 17. Oxaliplatin effect on Na_V currents of IB4(+) neurons. **A**) Representative recordings of the increased duration of the Na_V current detected at -30 mV for vehicle (blue) and oxaliplatin (red). **B**) J-V relationships of Na^+ inward currents present in IB4(+) neurons. J ($pA{\cdot}pF^{-1}$) median values with interquartile range are shown. Mann-Whitney test. Right G-V curves obtained from the J-V curves. Reversal potential was interpolated from each J-V curve. Curves were fitted to the Boltzmann equation: $G/G_{max} = \left(1/(1+e^{-\frac{zF(V_{1/2}-V)}{RT}})\right)$. Data from neurons treated with vehicle are blue coloured, and with oxaliplatin (OHP) red coloured. **C-D**) Fast (**C**) and slow (**D**) time constant of inactivation (τ_{fast}) at different voltage pulses for IB4(+) neurons after vehicle or oxaliplatin treatment. Inactivation curves were fit to a double exponential equation. **E**) Recovery from inactivation of the Na_V currents against time for IB4(+) neurons. Unpaired t-test between vehicle and oxaliplatin treated groups. *P*-values for statistically significant differences are indicated in each graph.

Then, we performed different protocols to determine which Na_V channel subtype could be altered by oxaliplatin. First, the $Na_V1.6$ current was blocked using its specific antagonist 4,9-anhydro-TTX. The resulting current was subtracted from the total Na_V current to isolate the portion corresponding to the $Na_V1.6$ channel activity. When we plotted the current density, we could not observe any significant augment in its amplitude (Figure 18A, C). The tendency to slow the current inactivation (τ_{fast}) after oxaliplatin treatment was also maintained in the presence of the blocker (Figure 18D), suggesting an additional mechanism to explain the effect observed in Figure 17C. Next, we

applied a voltage protocol previously designed for isolating $Na_V1.8$ current responses (Soriano et al., 2019). As a result, there was a tendency to increase $Na_V1.8$ current density 0 h post-treatment with oxaliplatin (Figure 18B, E). The $Na_V1.8$ current inactivation kinetics seemed to slightly increase after oxaliplatin treatment (vehicle: 1.5 (1.3-2.3) ms, oxaliplatin 2.1 (1.5-2.2) ms), although no significant alterations were observed (Figure 18F).

Investigation of the mRNA expression of the main Na_V channels did not result in any significant difference. Nonetheless, we could notice higher $Na_V1.6$ relative expression after oxaliplatin treatment in 4 out of 5 independent cultures analyzed (vehicle: 4 ± 4, oxaliplatin: 8 ± 7 ($\cdot 10^{-3}$ a.u.)) (Figure 26A). The lack of differences observed with the $Na_V1.6$ blocker 4,9-anhydro-TTX could be explained by oxaliplatin mediated transcriptional modification of the channel on different populations of the culture, such as medium or large size DRG neurons.

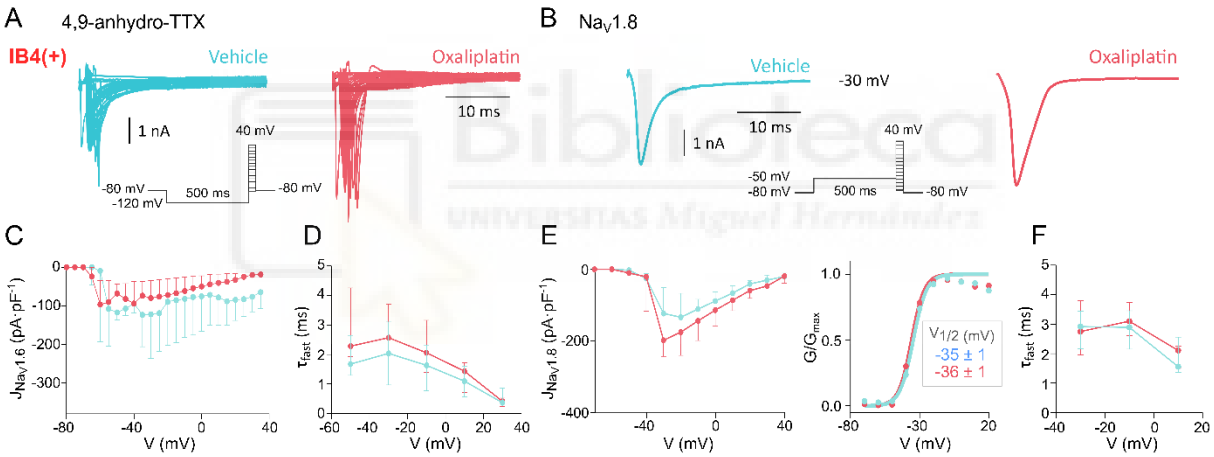


Figure 18. Oxaliplatin effect on $Na_V1.6$ and $Na_V1.8$ activities of IB4(+) neurons. **A**) Pharmacological dissection of vehicle and oxaliplatin Na_V currents using the $Na_V1.6$ specific blocker 4-anhydro-TTX at 50 nM. The current after blockage was subtracted from the total current measured and the resultant representative traces are shown. **B**) Representative recordings of the $Na_V1.8$ current isolated with the specific voltage steps protocol measured at -30 mV. **C**) J-V relationship of the $Na_V1.6$ current subtracted. J (pA·pF^{-1}) median values with interquartile range are shown. **D**) Inactivation time constant (τ_{fast}) of the total Na_V current for vehicle and oxaliplatin conditions under application of 4,9 AnhydroTTX. **E**) JV (left) and GV (right) curves for $Na_V1.8$ current. $V_{1/2}$ values are indicated inside the square. **F**) τ_{fast} for the $Na_V1.8$ current at -30, -10 and 10 mV after vehicle or oxaliplatin treatment. Data from neurons treated with vehicle are blue coloured, and neurons treated with oxaliplatin are red coloured. Data are expressed as median (IQR). Data were collected 0 h post-treatment. Mann-Whitney test.

Oxaliplatin altered K_V currents on IB4(+) neurons

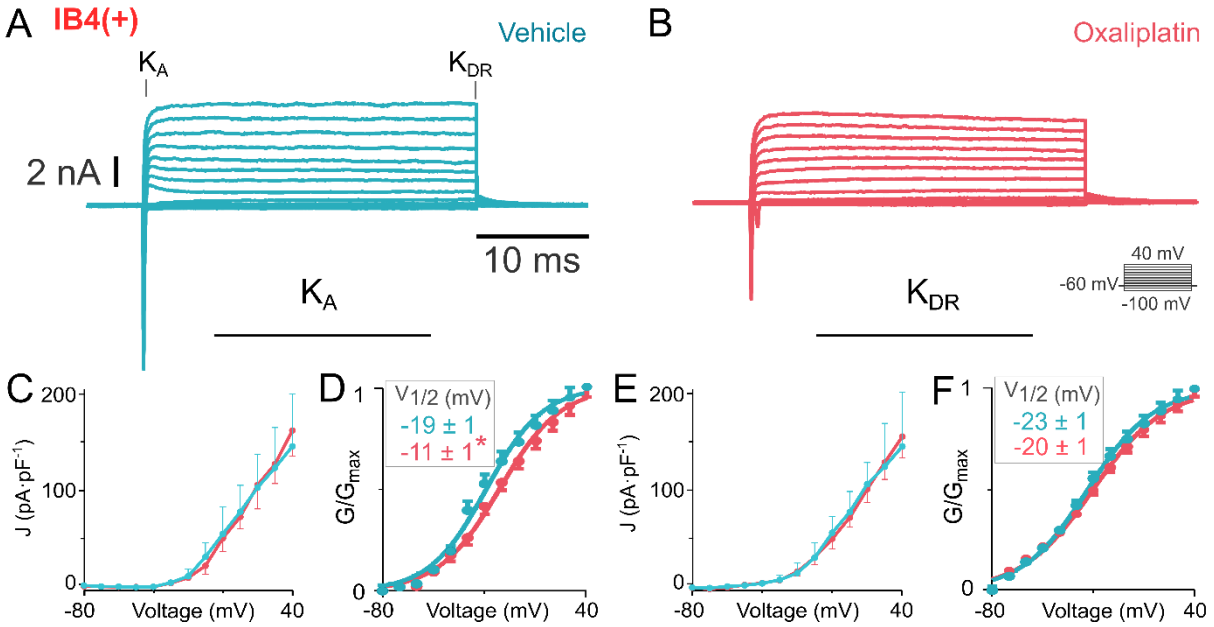


Figure 19. Oxaliplatin effect on K_V current of IB4(+) neurons 0 h post-treatment. A, B) Representative recordings of K_V current in response to voltage steps from -100 to 40 mV in 10 mV intervals. **C, E**) Current density (median (IQR)) curve of IB4(+) neurons exposed to vehicle (blue) or oxaliplatin (red) for K_A (**C**) and K_{DR} (**E**). **D, F**) G/G_{max} curve for K_A (**D**) and K_{DR} (**F**) of IB4(+) neurons. Data are represented as mean ± SEM. Mann-Whitney test. $V_{1/2}$ values are shown as mean ± SD and compared with an Unpaired t-test. **P* < 0.05, for significant differences between vehicle and oxaliplatin groups.

Then, as an important contributor to the nociceptor excitability, we decided to analyze the activity of the voltage-gated potassium channels (K_V) in IB4(+) neurons. To do this, we applied a voltage steps protocol from -100 to 40 mV in 10 mV intervals. At each voltage applied, we measured the outward current density evoked 0 h post-treatment with vehicle or oxaliplatin (Figure 19A-B). We analyzed separately two distinct potassium currents: the fast-adapting K_A and the delayed rectifying K_{DR}. Under these conditions, the current density measured at each voltage was not significantly affected by oxaliplatin (Figure 19C, E). However, the conductance-voltage relationship was altered. As shown with the $V_{1/2}$ values, oxaliplatin exposure shifted the activation curve of K_A current 8 mV to more positive potentials, implicating that more depolarization is needed to activate these channels (Figure 19D). K_{DR} showed a small positive shift of 3 mV after treatment (Figure 19F).

Oxaliplatin effect on male and female DRG neurons

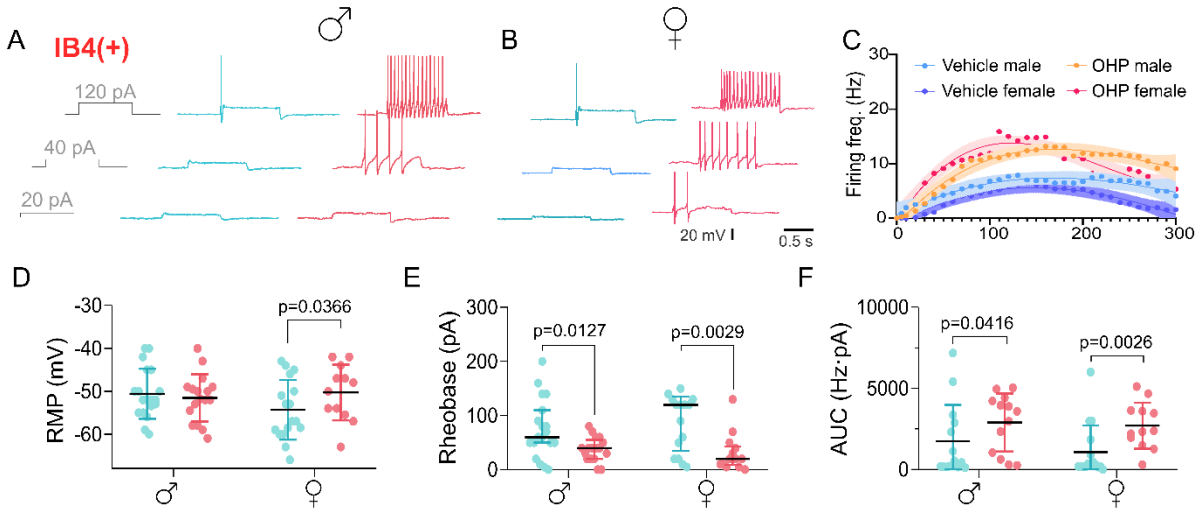


Figure 20. Male and female IB4(+) DRG neurons excitability 0 h after vehicle or oxaliplatin treatment. A, B) Representative voltage traces in response to 20 pA, 40 pA and 120 pA current injection for male (**A**) and female neurons (**B**). **C**) Firing frequency as a function of the injected current in the range of 0 to 300 pA in 10 pA pulses for male and female neurons. Data from neurons treated with vehicle are coloured in light blue for males and dark blue for females, and those from neurons treated with oxaliplatin (OHP) with orange for males and red for females. Firing frequency was obtained counting the number fired of APs during the 1 s current pulse. **D**) RMP measured using a continuous current protocol of 0 pA in female and male neurons. Data are presented as individual values (dots) and the mean (black lines) with standard deviation (blue or red lines). **E**) Rheobase values estimated as the minimum injected current need to fire an action potential in female and male neurons. **F**) Area under the curve (AUC_0^{300}) obtained by integrating the firing frequency curve for the 0-300 pA current interval in female and male neurons. Data were collected 0 h post-treatment. Unpaired t-test for RMP and Mann-Whitney test for rheobase and AUC. *P*-values shown indicate significant statistical differences.

An individual sex has shown a strong influence in numerous chronic pain disorders (Cabañero et al., 2022). In order to detect potential sex differences in oxaliplatin response, primary nociceptor cultures obtained from male and female rats were analyzed separately immediately after treatment. In Figure 20D it is possible to appreciate that oxaliplatin treatment strongly depolarized female DRG neurons. Of note, RMP from male and female vehicle groups also differed, showing a more hyperpolarized value on females. Reduced rheobase and AUC was observed in both sexes, although a more prominent effect compared to the vehicle can be appreciated in female neurons (Figures 20E, F).

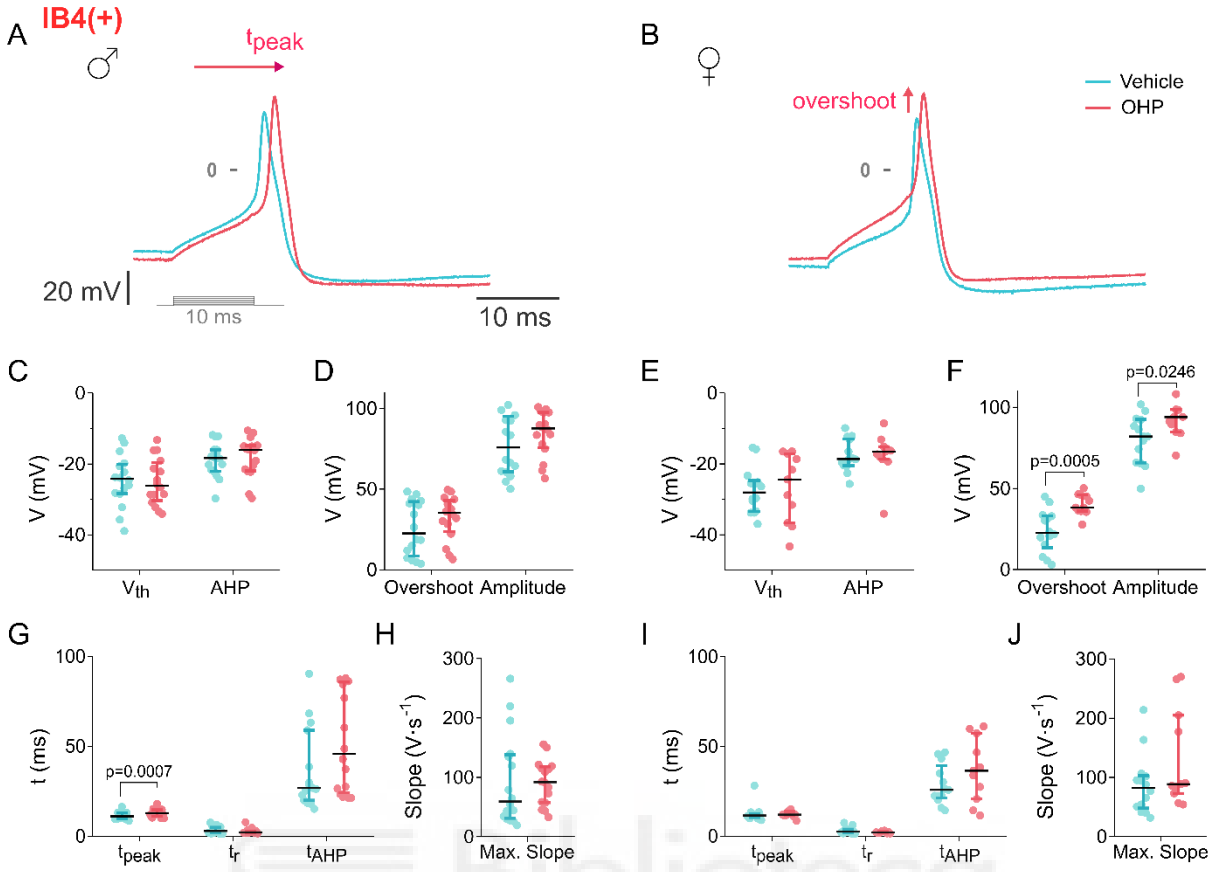


Figure 21. AP parameters for male and female IB4(+) neurons 0 h post-treatment. A, B) Representative AP recordings for male (**A**) and female (**B**) DRG neurons after vehicle (blue) or oxaliplatin (red) exposure. **C, E)** Action potential threshold (V_{th}) and AHP recorded for male (**C**) and female (**E**) neurons. **D, F)** Overshoot and Amplitude for male (**D**) and female (**F**). **G, I**) Time to peak (t_{peak}), repolarization time (t_r) and t_{AHP} parameters for male (**G**) and female (**I**) neurons. **H, J**) Maximum upstroke slope for male (**H**) and female (**J**) neurons. Unpaired t-test for t_{AHP}, Overshoot, Amplitude, V_{th} and AHP**.** Mann-Whitney test for t_{peak}, t_r and Max. Slope. Statistically significant ($P < 0.05$) values between vehicle and oxaliplatin groups are indicated in each graph.

Regarding the individual action potential parameters, cultures obtained from female rats showed a significant increase in the overshoot and amplitude of the AP (overshoot: vehicle: 23.7 ± 12.9, oxaliplatin: 40.2 ± 6.5 mV; amplitude: vehicle: 80.1 ± 14.8, oxaliplatin: 94.0 ± 13.1 mV) (Figure 21F). These parameters also had a tendency to augment in male cultures, but no significant differences were reached (Figure 21D). On the contrary, the time necessary to arrive to the peak of the AP from the beginning of the current stimulus was increased by oxaliplatin treatment only on male cultures (11.4 ± 2.0 to 13.0 ± 2.2 ms) (Figure 21G, I).

Sexual dimorphism in the electrogenicity of DRG neurons could be explained by a differential regulation of the voltage-gated dependent ion channels Na_V and K_V. Thus, we first analyzed the Na_V current response to depolarizing voltage pulses applying the protocols described in Figure 17. As a result, we could observe that oxaliplatin reduced the Na_V current density on male rat cultures without altering the conductance of the channels (Figure 22A, B, E, F). These currents were also slower than in vehicle conditions since the time constant of inactivation increased at -30, -10 and 10 mV (Figure 22E).

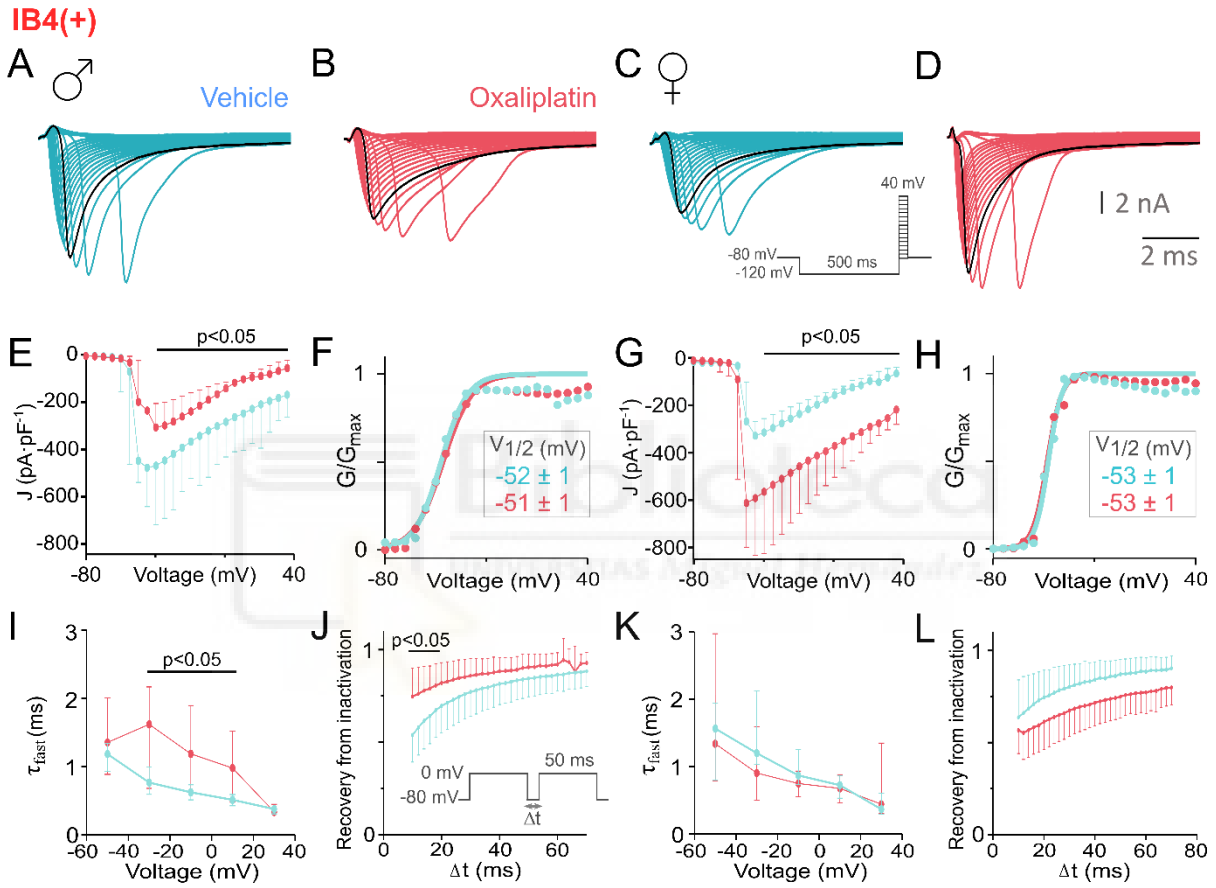


Figure 22. Oxaliplatin differentially altered Na_V current of male and female IB4(+) neurons 0 h post-treatment. A-D) Representative recordings of Na_V current in response to the voltage protocol applied in Figure 17A for male (**A, B**) or female (**C**, **D**) IB4(+) neurons. The current elicited at -30 mV is in black. **E, G**) Current density-voltage curve of IB4(+) neurons exposed to vehicle (blue) or oxaliplatin (red) for male (**E**) and female (**G**). **F, H**) G/G_{max} curve for male (**F**) and female (**H**) IB4(+) neurons. Data are represented as mean ± SEM. $V_{1/2}$ values are shown as mean ± SD. **I, K**) Fast time constant of inactivation (τ_{fast}) at different voltage pulses for male (**I**) and female (**K**) IB4(+) neurons after vehicle or oxaliplatin treatment. Inactivation curves were fit to a double exponential. **J, L**) Recovery from inactivation of the Na_V currents against time for male (**J**) and female (**L**) IB4(+). Unpaired t-test. $P < 0.05$ for significant differences between vehicle and oxaliplatin groups is indicated.

Furthermore, oxaliplatin treated neurons had Na_V currents that recovered faster from inactivation at t ≤ 24 ms between pulses (Figure 22J). For DRG neurons obtained from female rats, we could not find alterations in the Na_V current inactivation kinetics nor in the recovery from inactivation (Figure 22K, L). However, we could detect a significant increase in the Na_V current density from -40 to 35 mV (J at -40 mV for female: vehicle: -315 ± 65 pA·pF^{-1}, oxaliplatin: -591 ± 230 pA·pF^{-1}) (Figure 22G).

When we analyzed the $Na_V1.8$ activity, we observed similar current amplitudes between male and female rat cultures (Figures 23A, C). These results suggest that the reduced current density observed in male rat cultures could be produced by a different Na_V subtype. The conductance voltage relationship however, showed a shift to more negative potentials in male cultures (Figure 23B) and to more positive potentials in female cultures (Figure 23D).

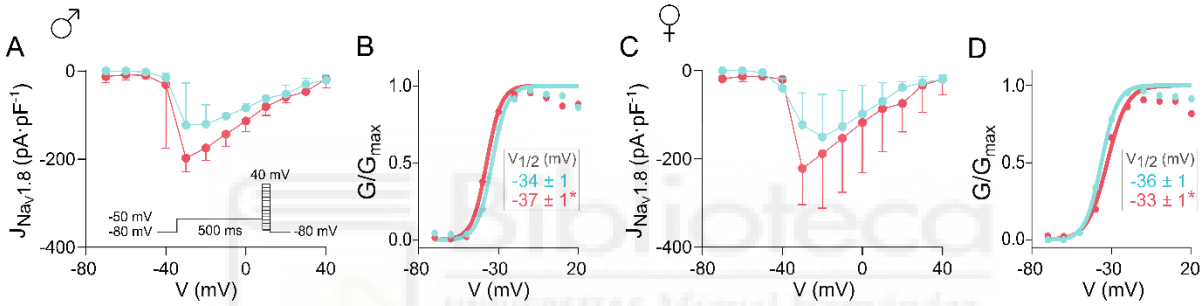


Figure 23. Oxaliplatin effect on $Na_V1.8$ current of male and female IB4(+) neurons 0 h post-treatment. A, C) J-V curve of IB4(+) neurons exposed to vehicle (blue) or oxaliplatin (red) for male (**A**) and female (**C**) obtained with the protocol shown in Figure 18B for isolating $Na_V1.8$ currents. **B, D**) G-V curve for male (**B**) and female (**D**) IB4(+) neurons. Data are represented as mean ± SEM. $V_{1/2}$ values are shown as mean ± SD inside the squares. Unpaired t-test. *P < 0.05, for significant differences between vehicle and oxaliplatin groups.

The potential sex differences on the K_V channels behavior were investigated separating male and female DRG cultures. As a result, a major effect was observed on the K_A activity of female neurons. Oxaliplatin reduced the current density of K_A at -10 mV only in female neurons (Figure 24E, G). It was also capable of eliciting a strong shift in the $V_{1/2}$ values of 11 mV to more depolarized potentials (Figure 24H). Male neurons exhibited a smaller depolarizing shift of 4 mV (Figure 24F). For K_{DR}, a similar current response was observed in vehicle and oxaliplatin treated cells of male neurons, meanwhile a significant 6 mV shift to higher voltages was observed in female nociceptors (Figure 24I-L).

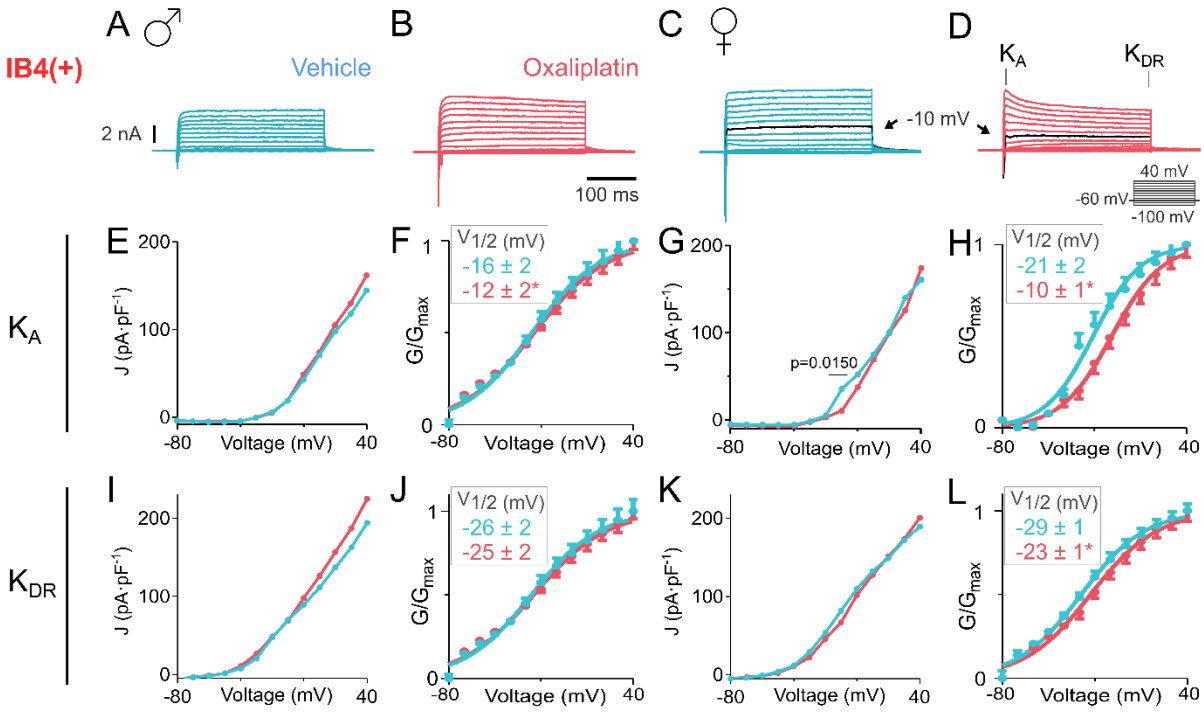


Figure 24. Oxaliplatin reduced K_A current of IB4(+) neurons 0 h post-treatment on female cultures. A-D) Representative recordings of K_V current in response to different voltage pulses. **E, G, I, K**) J-V curve of IB4(+) neurons exposed to vehicle (blue) or oxaliplatin (red) for male (**E**) and female (**G**) K_A and male (**I**) and female (**K**) K_{DR}. **F, H, J, L**) G-V curve for male (**F**) and female (**H**) K_A and male (**J**) and female (**L**) K_{DR} of IB4(+) neurons. Data are represented as mean ± SEM. J values at each voltage were analyzed with a Mann-Whitney test. $V_{1/2}$ values are shown as mean ± SD and statistically analyzed with an Unpaired t-test. $*P < 0.05$, for significant differences between vehicle and oxaliplatin groups is indicated.

Oxaliplatin increased TRPV1 and TRPA1 activities

TRP channels have been hypothesized to play a pivotal role on CIPN pain symptoms (Cabañero et al., 2022). Therefore, we investigated the TRPV1, TRPA1 and TRPM8 functional changes using microelectrode arrays (MEA) at the peak of excitability (0 h post-treatment). As a result, the firing frequency significantly augmented in response to the TRPV1 agonist capsaicin (from 0.2 (0.5-0.1) to 0.4 (4-0.1) Hz) and the TRPA1 agonist AITC (from 0.1 (0.4-0.04) to 0.3 (2-0.06) Hz) after oxaliplatin treatment, whereas menthol (TRPM8 agonist) responses were similar in both untreated and treated groups (Figure 25A, C). The percentage of electrodes responding to AITC showed a tendency to increase after oxaliplatin treatment from 50 to 70 % (calculated respect to the total active electrodes), although no significant results were obtained in the Fisher's exact test (Figure 25B).

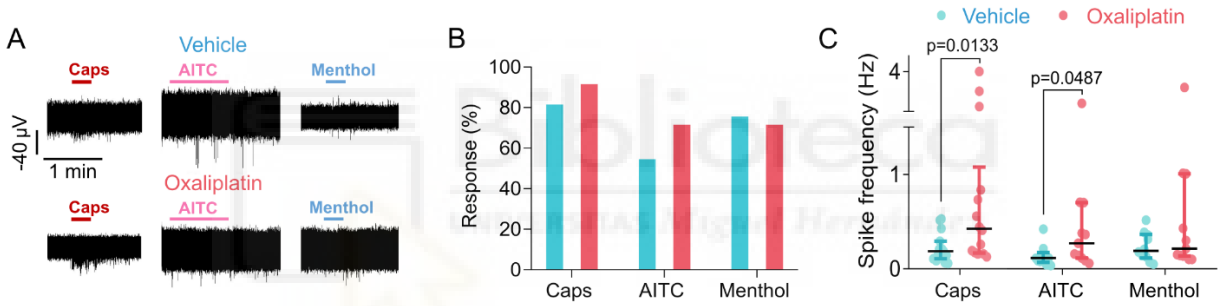


Figure 25. Increased capsaicin and AITC responses 0 h after oxaliplatin removal on microelectrode arrays (MEA). (**A**) Representative MEA recordings performed 0 h after vehicle or oxaliplatin treatment (DIV6). The protocol followed consisted of two applications of 20 s of capsaicin (Caps) at 1 µM, application of 60 s of allyl isothiocyanate (AITC) at 100 µM, and application during 20 s of menthol at 100 µM. (**B**) Percentage of electrodes with response to Caps, AITC or menthol respect to the total active electrodes. Fisher's exact test. (**C**) Spike frequency of the responses to Caps, AITC and menthol. Dots correspond to the values measured for each electrode and lines represent the median (IQR). Mann-Whitney test. *N* = 3, number of MEA plates = 6. Number of units = 18 for vehicle, 15 for oxaliplatin group. *P*-values indicating significant statistical differences between vehicle and oxaliplatin groups are indicated in the graphs.

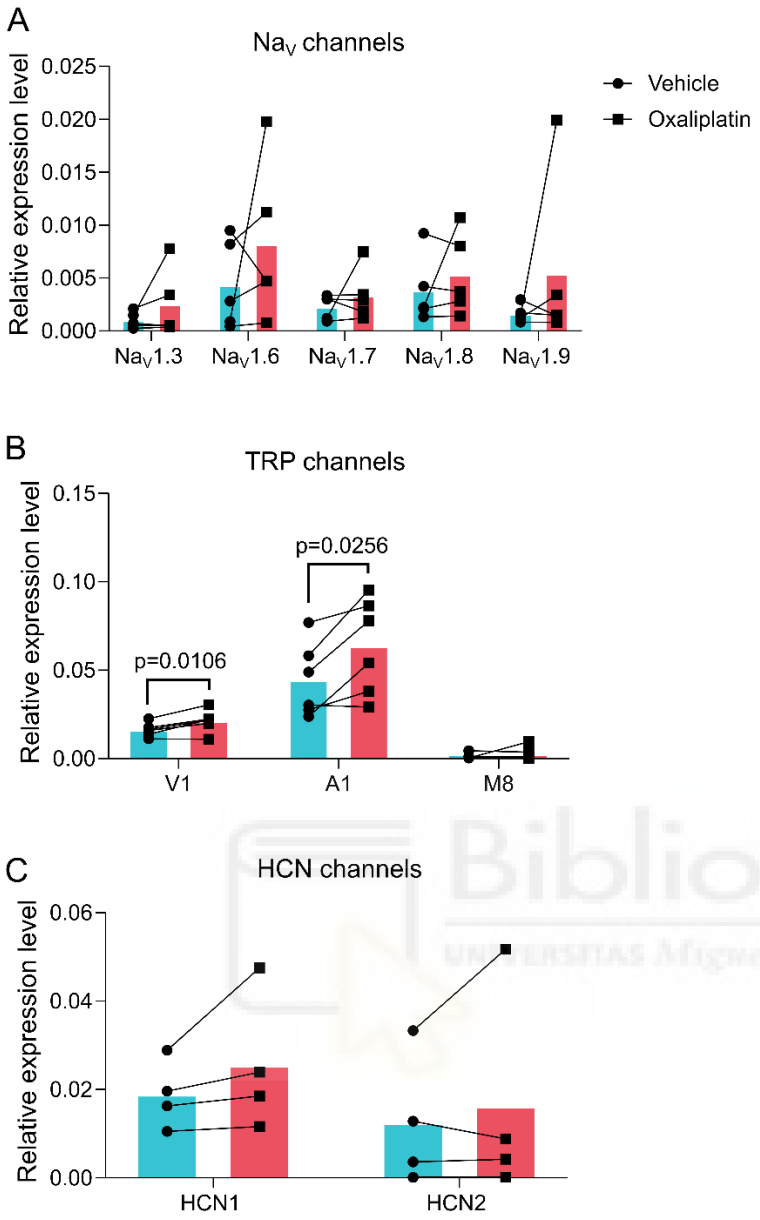


Figure 26. Oxaliplatin exposure increased TRPV1 and TRPA1 mRNA expression. Measured mRNA levels of the different Na$_V$, TRP and HCN channels 0 h post-treatment. **A-C)** Quantification of the mRNA levels of Na$_V$ **(A)**, TRP **(B)** and HCN **(C)** channels 0 h after vehicle (blue) and oxaliplatin exposure (red) (DIV 6). Each dot shows the mRNA level of each independent culture. Dots showing vehicle and oxaliplatin values from the same culture relate to a line. Relative expression indicates the mRNA level of each channel normalized to the internal control Rpl29. Paired t-test. *P*-values for statistically significant differences are shown.

Then, we hypothesized that the altered TRPV1 and TRPA1 activity could be the result of increased ion channel gene expression. To test this hypothesis, we measured the mRNA levels of TRPV1, TRPA1 and TRPM8 ion channels. In Figure 26B, we could observe that oxaliplatin significantly increased the mRNA expression of TRPV1 and TRPA1 ion channels. TRPV1 expression augmented from 16 ± 4 to 21 ± 6 a.u., and TRPA1 increased from 44 ± 21 to 64 ± 27 a.u. (Table 6, Figure 26B). We could not find significant changes on TRPM8 mRNA expression (Table 6, Figure 26B). These findings together with the MEA results suggested a relevant contribution of TRPV1 and TRPA1 on oxaliplatin-induced neuropathy.

Table 6. mRNA expression levels of different ion channels after vehicle or oxaliplatin exposure (0 h post-treatment).

	mRNA expression ($x10^{-3}$)		
Ion channel	**Vehicle**	**Oxaliplatin**	***N***
TRPV1	16 ± 4	21 ± 6*	6
TRPA1	44 ± 21	64 ± 27*	6
TRPM8	1 ± 2	3 ± 4	6
HCN1	19 ± 8	25 ± 16	4
HCN2	12 ± 15	16 ± 24	4
$Na_V1.3$	1 ± 1	3 ± 3	5
$Na_V1.6$	4 ± 4	8 ± 7	5
$Na_V1.7$	2 ± 1	3 ± 2	5
$Na_V1.8$	4 ± 3	5 ± 4	5
$Na_V1.9$	2 ± 1	5 ± 8	5

*P-value < 0.05. Paired t-test. N: number of independent cultures/animals.

Additionally, we investigated the expression levels of HCN1 and HCN2 ion channels that were also associated with oxaliplatin-induced neuropathy. In the four independent cultures examined, an upregulation tendency of HCN1 was detected after oxaliplatin treatment, suggesting a potential participation to the exacerbated excitability found under this condition (Figure 26C, Table 6).

We continued investigating the current activity of TRPV1, TRPA1 and TRPM8 on the IB4(-) and IB4(+) small populations of DRG neurons immediately after vehicle or oxaliplatin treatment. IB4(+) neurons showed a significant augment in the current density evoked after capsaicin application (Table 7, Figure 27A, G), indicating a potentiation of TRPV1 activity elicited by oxaliplatin treatment (capsaicin median response: vehicle: 3 (2-9) $pA{\cdot}pF^{-1}$, oxaliplatin: 10 (7-12) $pA{\cdot}pF^{-1}$). TRPA1 current density showed

a tendency towards a 2-fold increase in the median activity despite not finding significant differences due to the lower number of responding cells in the vehicle group (Tables 7-8, Figure 27B, H). Indeed, we found a significant higher percentage of neurons responding to AITC rising from 30 to 74 % between vehicle and oxaliplatin groups (Table 8, Figure 27E). No differences were detected in the percentage of neurons expressing TRPM8 nor in its current density after treatment (Tables 7-8, Figure 27C, F, I).

IB4(-) did not experience any significant alteration on the current density of these TRP channels when their agonists were applied (Figure 27A-C, G-I). This also occurred with the percentage of the neurons recorded responding to these compounds, although a small tendency to increase the number of neurons with response to capsaicin and AITC could be noted (Figure 27D-F, capsaicin: 89 % vehicle, 100 % oxaliplatin; AITC: 25 % vehicle, 44 % oxaliplatin). However, there was a tendency to increase the capsaicin-evoked current density by 2-fold at 96 h post-treatment (Table 7, Mann-Whitney test, $P = 0.0506$).

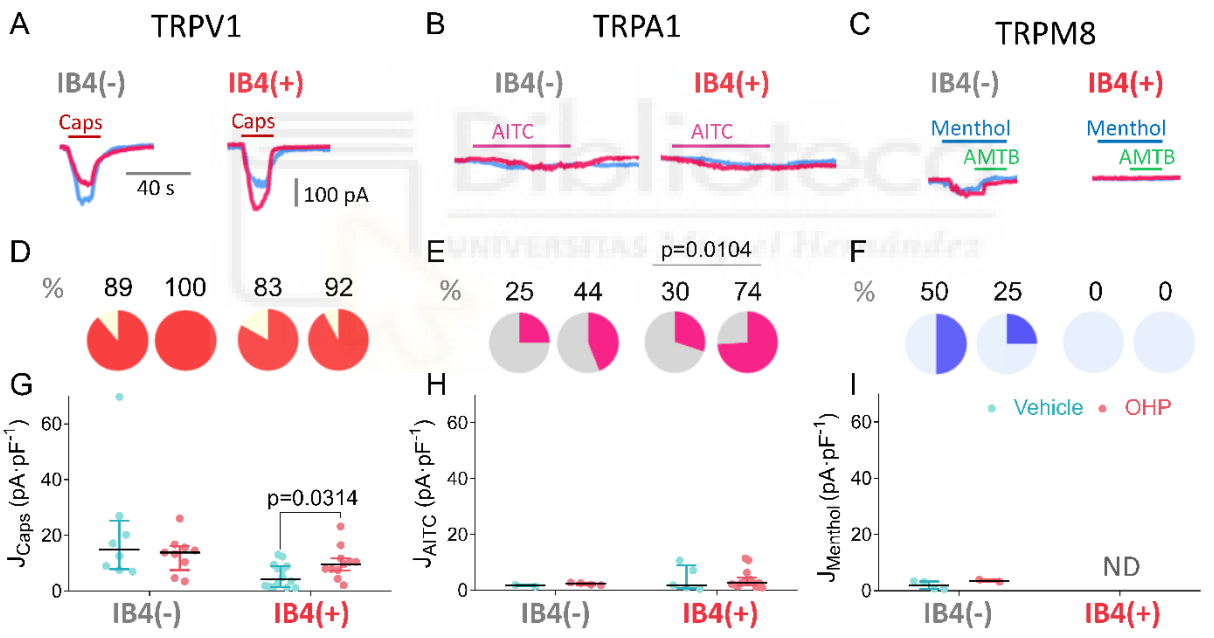


Figure 27. Oxaliplatin increased TRPV1 and TRPA1 activities 0 h post-treatment in IB4(+) neurons. A-C) Representative recordings of the current responses elicited by application of capsaicin (Caps) (**A**), AITC (**B**), and menthol (**C**) 0 h after vehicle or oxaliplatin treatment removal for IB4(-) and IB4(+) neurons. AMTB was used as a control of TRPM8 currents. **D-F)** Percentage of cells with response to Caps (**D**), AITC (**E**) or menthol (**F**) respect to the total of cells registered for IB4(-) (left) and IB4(+) (right) neurons. Fisher's exact test. **G-I**) Current density values for IB4(-) and IB4(+) neurons exposed to vehicle (blue) or oxaliplatin (red) in response to capsaicin (**G**), AITC (**H**) and menthol (**I**). Data are represented as mean ± SD. Unpaired t-test between vehicle and oxaliplatin groups. *P*-values for significant statistical differences are indicated in the graphs.

Table 7. TRPV1, TRPA1 and TRPM8 current densities after oxaliplatin exposure.

		0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
		Vehicle	Oxaliplatin	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin
TRPV1	IB4(-)	15 (8-26)	14 (9-16)	20 (8-43)	13 (6-29)	14 (3-100)	33 (11-37)
	IB4(+)	3 (2-9)	10 (7-12)* p=0.0314	3 (1-4)	6 (3-9)	7 (5-8)	6 (5-13)
TRPA1	IB4(-)	2 (1-2)	2 (2-3)	2 (1-2)	8 (3-9)	2 (1-4)	2 (2-2)
	IB4(+)	2 (1-9)	3 (2-5)	2 (1-5)	5 (3-7)	2 (1-4)	5 (1-17)
TRPM8	IB4(-)	2 (1-3)	4 (3-4)	3 (1-14)	7 (5-8)	2 (1-3)	2 (0-2)
	IB4(+)	ND	0.5	ND	ND	ND	ND

Current densities for each channel measured at 0 h (DIV 6), 48 h (DIV 8) and 96 h (DIV 10) post-treatment for IB4(-) and IB4(+) DRG neurons. Values are expressed in $pA \cdot pF^{-1}$. Vehicle and Oxaliplatin were compared using the Mann-Whitney test for TRPA1, and the Unpaired t-test for TRPV1 and TRPM8. *$P < 0.05$. Data are expressed as median (Q25-Q75). ND: non-detected.

Table 8. Percentage of the cells registered responding to the TRPV1, TRPA1 and TRPM8 agonists after vehicle and oxaliplatin exposure.

		0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
		Vehicle	Oxaliplatin	Vehicle	Oxaliplatin	Vehicle	Oxaliplatin
TRPV1	IB4(-)	89 % 8/9	100 % 11/11	100 % 6/6	63 % 5/8	50 % 4/8	70 % 7/10
	IB4(+)	92 % 12/13	92 % 12/13	80 % 4/5	75 % 6/8	75 % 6/8	70 % 7/10
TRPA1	IB4(-)	25 % 2/8	44 % 4/9	23 % 3/13	40 % 4/10	60 % 6/10	22 % 2/9
	IB4(+)	30 % 6/20	74 %* 14/17 p=0.0104	78 % 7/9	100 % 10/10	73 % 8/11	79 % 11/14
TRPM8	IB4(-)	50 % 4/8	25 % 2/8	38 % 5/13	22 % 2/9	33 % 3/9	38 % 3/8
	IB4(+)	0 % 0/18	6 % 1/17	0 % 0/9	0 % 0/10	0 % 0/11	0 % 0/14

Percentage of cells showing a current response to capsaicin (TRPV1), AITC (TRPA1), or menthol (TRPM8) in a continuous voltage protocol at -60 mV. Data measured at 0 h (DIV 6), 48 h (DIV 8), and 96 h (DIV 10) post-treatment for IB4(-) and IB4(+) DRG neurons. The number of neurons that responded to each TRP channel agonist respect to the total number of neurons measured for each condition is indicated below. Vehicle and Oxaliplatin were compared using the Fisher's exact test. *$P < 0.05$.

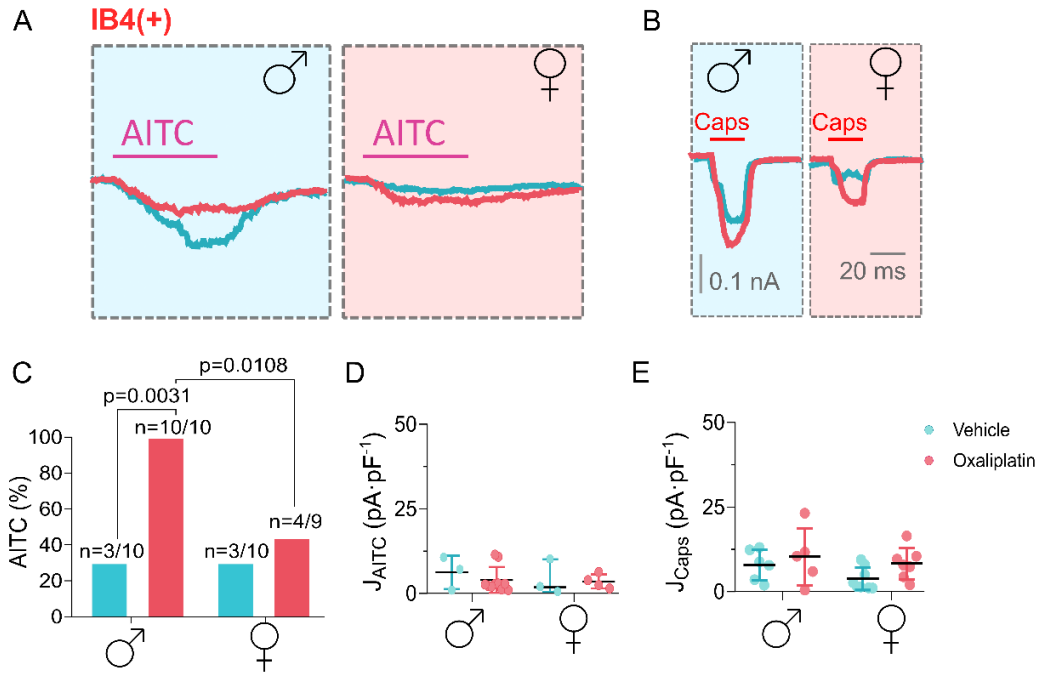


Figure 28. TRPV1 and TRPA1 activities in male and female IB4(+) DRG neurons 0 h after vehicle or oxaliplatin. A, B) Representative recordings of the current responses evoked in cells that responded to application of AITC (**A**) and capsaicin (**B**) for vehicle (blue) and oxaliplatin treated neurons (red). **C**) Percentage of neurons that responded to the TRPA1 agonist AITC at 100 µM. Fisher's exact test. **D, E**) Peak current density observed after application of AITC (**D**) and capsaicin 1 µM (**E**). Kruskal-Wallis with Dunn's multiple comparisons test. $P < 0.05$, indicating significant statistical differences.

Recent research also pointed towards a pivotal role of TRP channels on CIPN sexual dimorphism due to its modulation by sex hormones and the finding of sex differences in the TRP pathways (Cabañero et al., 2022). Since we have found a tendency of higher excitability on female neurons, we analyzed if there were potential sex differences on TRP channels activity (Figure 28).

As a result, 0 h after removing oxaliplatin, the percentage of neurons with response to AITC was significantly higher for male (100 %) than for female (44 %) IB4(+) neurons (Figure 28C). This augment in the response was not translated into an increase in the current density evoked by AITC (Figure 28A, D). No significant differences were reached when the current density in response to capsaicin was analyzed (Figure 28B, E). However, a strong tendency to augment capsaicin responses could be observed in both male and female cultures (Figure 28B, E).

ThermoTRP channels in pain sexual dimorphism

ThermoTRP channels in pain sexual dimorphism: new insights for drug intervention

Results obtained from Publication 2

The review that we performed on the potential role of thermoTRP channels in pain sexual dimorphism and its therapeutic potential was published in the journal *Pharmacology & Therapeutics*:

Publication 2.

- Cabañero, D.*, Villalba-Riquelme, E.*, Fernández-Ballester, G., Fernández-Carvajal, A. & Ferrer-Montiel, A. (2022). ThermoTRP channels in pain sexual dimorphism: new insights for drug intervention. *Pharmacology & Therapeutics*, 240, 108297, https://doi.org/10.1016/j.pharmthera.2022.108297 IF: 13.4 (Q1) *These authors contributed equally.

Introduction

Migraine and pain evoked by CIPN constitute two major types of chronic pain with increasing clinical relevance. Pharmacological management is still a challenge prescribing analgesic drugs with poor efficacy for the pain symptoms. Cumulative evidence points towards a crucial role for thermoTRP channels in these pathologies. Noteworthy, its modulation by sex hormones suggests their contribution to the sexual dimorphism experienced in many chronic pain syndromes. Thus, thermoTRP channels emerge as promising therapeutic tools for controlling the pain symptoms satisfactory in women and men. To address this issue, in this review, we focused on examining the sexual dimorphism, and the role and therapeutic potential of thermoTRP ion channels on migraine and CIPN.

Sexual dimorphism of chronic migraine

Migraine is a complex neurological disorder characterized by disabling recurrent headache attacks (Ferrari et al., 2022). It is estimated to affect 1 out of 7 people, being one of the most prevalent diseases around the world (Safiri et al., 2022). However, it does not affect equally all population groups since sex has been considered a migraine risk associated factor. Before puberty and after 50 years old, the incidence is similar in both sex, but when migraine reaches peak levels at the age of ~40 years old, migraine is experienced by 35% of women and 15% of men (Stovner, 2018).

For the people affected, migraine cause a drastic impact in their quality of life. Headache crises are painful and invalidating and are usually accompanied by photophobia, phonophobia, hypersensitivity to touch and/or nausea and vomiting (Stovner, 2018). This phase can be present between 4 to 72h but it also starts with prodromal symptoms that can also last for several days including fatigue, drowsiness, nausea and vomiting, yawning, mental slowness, impaired concentration, or photophobia. After the headache crisis, it can continue with postdromal symptoms that can also last for several days and is mainly characterized by tiredness, hyperesthesia or allodynia, somnolence, and a certain cognitive dysfunction (Ferrari et al., 2020; Louter et al., 2013).

To minimize the pain symptoms, NSAIDs or paracetamol are prescribed as first-line pharmacological treatment (Eigenbrodt et al., 2021). Second and third-line treatments include triptans and gepants, respectively. In the review, we noted that most of the novel therapeutics that are being tested for migraine, altogether with the gepants family of compounds, are targeting the αCGRP signaling pathway. As a result, this molecular pathway is emerging as a key mechanism in migraine. In trigeminal nociceptors, αCGRP is released after activation of thermoTRP channels (Devesa et al., 2014; Meng et al., 2009). Therefore, these ion channels are suggested as promising therapeutic opportunities for migraine and its sexual dimorphism.

TRP channels in the pathophysiology of migraine

The thermoTRP channels that have been mainly linked to migraine are TRPV1, TRPA1 and TRPM8. These channels are expressed in the trigeminal nerves where they are presumed to initiate the migraine attacks (Charles & Brennan, 2010). The specific contribution of each of these ion channels in migraine and its sexual dimorphism is detailed in the review. Here, I provide a summary of the most relevant findings on these channels.

TRPV1

TRPV1 has been directly related to the presence of migraine in humans since several single-nucleotide polymorphisms in its gene were associated with migraine (Carreño et al., 2012; Yakubova et al., 2021). Importantly, Gazerani et al. (2005) showed that application of capsaicin evoked stronger trigeminal sensitization in women than men, intensifying these symptoms with the menstrual phase. With this regard, modulation of TRPV1 by estrogens has been proposed in different regulatory pathways of the channel, emerging as a key player for treating migraine (Artero-Morales et al., 2018; Payrits et al., 2017). Indeed, reducing TRPV1 activity with botulinum toxins or desensitizing the channel with specific agonists were able to ameliorate migraine (Burstein et al., 2020; Fernández-Carvajal et al., 2020). However, TRPV1 is also present in brain areas related to pain with pronociceptive or

antinociceptive effects (Barrière et al., 2020; Xiao et al., 2016). Therefore, special caution should be taken when using drugs directed to TRPV1 for migraine.

TRPA1

Pre-clinical models of migraine have shown that TRPA1 was essential in mediating the pro-nociceptive effects of nitric oxide (NO) and reactive oxygen species (ROS) (De Logu et al., 2022; Marone et al., 2018; Sullivan et al., 2015). Indeed, the lack of TRPA1 avoided the pain experienced in male and female mice in a nitroglycerin migraine model (Alarcón-Alarcón et al., 2022). Despite being essential in both sexes, differences in TRPA1 convergent pathways such as increased prolactin circulating levels after stressful stimuli, a possible modulation of its expression by estrogens, and its interaction with female steroids could also explain the migraine sexual dimorphism (Fusi et al., 2014; Watanabe et al., 2022; Xie et al., 2022). Therefore, new clinical trials are being designed to analyze the therapeutic potential of TRPA1 antagonists, but no results are still available (clinicaltrials.gov, NCT05275751). Altogether, TRPA1 constitutes a promising therapeutic target for migraine.

TRPM8

Multiple TRPM8 polymorphisms have also been associated with migraine susceptibility (Chasman et al., 2011; Siokas et al., 2022). Stimulating TRPM8 with cool or menthol has been used by migraineurs to alleviate pain (Borhani Haghighi et al., 2010; St Cyr et al., 2015). This analgesic effect could be explained due to the inhibition caused on TRPV1/TRPA1 after its stimulation (Andersen et al., 2016; Gazerani et al., 2005; Ren et al., 2015). Importantly, an explanatory sexual dimorphic mechanism of TRPM8 was found in previous research in our laboratory, showing a protective effect of the channel by its activation with testosterone (Alarcón-Alarcón et al., 2022). On the contrary, other works suggested a pronociceptive effect of TRPM8 in migraine although limited results have been published on this line (Nahman-Averbuch et al., 2018). Due to its relevance in migraine pathophysiology, additional research was suggested to clarify the actions of TRPM8 in migraineurs and to help developing satisfactory treatments.

Other thermoTRP channels such as TRPV4, TRPV3 and TRPM3 are also showing a potential contribution in migraine, although the studies performed are limited and additional research on these receptors is suggested to fully understand migraine pathophysiology and its related sex differences (Carreño et al., 2012; Held et al., 2015; Mecklenburg et al., 2020; Wei et al., 2011).

Sexual dimorphism in chemotherapy-induced peripheral neuropathy

Due to the tight therapeutic window characteristic of chemotherapeutic drugs, special concerns are being paid to the presence of sexual dimorphism on CIPN (Wang et al., 2018). Small differences on its pathophysiology would result in increased toxicity, thus compromising patients' survival and quality

of life. Therefore, to analyze the current advances on this topic, we performed a bibliographic search of clinical and pre-clinical studies that analyzed CIPN in female and male subjects. We classified these results between the chemotherapeutic drugs administered in each study. Notably, for some clinical trials a combination of these drugs was prescribed to the patients, complicating this analysis. In some of them, patients were included by the type of tumor and the specific chemotherapy drugs provided were not disclosed. These results were collected in the section of combination of therapies in the same table (Table 1, Publication 2).

As a result, many of the studies evaluated are providing evidence on the existence of sex differences in CIPN mechanisms and symptoms. Among the most common clinical findings, CIPN incidence, sensitivity and/or severity was higher in women for most of the chemotherapeutic regimens (Table 1, Publication 2).

Thus, investigating the molecular mechanisms implicated would be essential for CIPN management. In order to analyze these mechanisms, we evaluated and elaborated a schematic representation of the potential mechanisms that might be involved in CIPN sex differences (Figure 3, Publication 2). Akin to migraine, the thermoTRP channels TRPV1, TRPA1 and TRPM8 seem to play a pivotal role on the sexual dimorphism of CIPN.

TRP channels in the pathophysiology of CIPN

TRPV1 has been found to be upregulated by numerous chemotherapeutic drugs including paclitaxel, docetaxel, oxaliplatin, cisplatin, bortezomib and vincristine (Chiba et al., 2017; Ertilav et al., 2021; Quartu et al., 2014; Ta et al., 2010; Villalba-Riquelme et al., 2022). As part of this doctoral thesis, we reproduced and confirmed the TRPV1 upregulation evoked by paclitaxel in our *in vitro* pre-clinical model (Publication 1) (Villalba-Riquelme et al., 2022). There, its alteration appears to have a crucial role since it has been linked to the thermal hyperalgesia and mechanical allodynia experienced by these patients.

TRPA1 alterations have also been commonly reported after the use of several chemotherapeutics such as oxaliplatin, cisplatin, carboplatin, thalidomide, and bortezomib (De Logu et al., 2020; C. Li et al., 2018; Liu et al., 2019). It has also been linked to ifosfamide visceral pain, and paclitaxel CIPN, although more information is needed to confirm these results (Materazzi et al., 2012; Pereira et al., 2013). The TRPA1 role on CIPN has been mainly attributed to the mechanical hyperalgesia experienced after oxaliplatin, bortezomib, and thalidomide (De Logu et al., 2020; C. Li et al., 2018; Liu et al., 2019). In addition, due to its contribution as a noxious cold detector, TRPA1 has been hypothesized to be responsible of the cold allodynia suffered by oxaliplatin patients (Zhao et al.,

2012). Nevertheless, alterations of the cool gated receptor TRPM8 have also been attributed to the oxaliplatin-induced cold allodynia.

Apart from its contribution to oxaliplatin-induced neuropathy, **TRPM8** mRNA expression was increased in an animal model of cisplatin neuropathy (Kawashiri et al., 2012). In our work, we could also observe enhanced TRPM8 activity and immunoreactivity after paclitaxel treatment, a finding that could explain the cold allodynia reported in these patients (Villalba-Riquelme et al., 2022).

TRP channels in CIPN sexual dimorphism

Importantly, novel research is finding different molecular pathways for male and female animals under CIPN. In the review, we elaborated a schematic representation of detected and potential mechanisms that could explain CIPN sexual dimorphism (Figure 3, Publication 2). There, TRP channels seem to play a pivotal role on it. With this regard, we found a significant higher increase in TRPM8 activity in male rats DRG cultures after paclitaxel treatment (Villalba-Riquelme et al., 2022). Furthermore, only in females, paclitaxel treatment produced mechanical pain through IL23/IL17/TRPV1 axis (Luo et al., 2021). This pathway was also activated by estradiol, a sex hormone whose levels were higher in the sciatic nerve of females (Luo et al., 2021). Estrogens were also implicated in the greater mechanical hyperalgesia experienced by females in this model, with results suggesting that TRPV1 could be implicated in signaling this effect (Luo et al., 2021). Despite some studies indicating estrogen agonists as pain relievers, most of the research analyzed signaled in the opposite direction with estrogen antagonists being antinociceptive (Ma et al., 2016; Paller et al., 2009).

Other sexual hormones such as progesterone and its derivatives or testosterone also seemed to modulate the pain derived from CIPN. For progesterone and its derivatives, a general antinociceptive effect for the pain induced by several chemotherapeutic treatments such as cisplatin, oxaliplatin, docetaxel, and vincristine has been reported (Meyer et al., 2010, 2011; Roglio et al., 2009; Zaki et al., 2018). Since progesterone was able to downregulate TRPV1, TRPA1, and TRPV4, this protective effect has been attributed to the alteration of these thermoTRP channels (Jung et al., 2009; Ortíz-Rentería et al., 2018). The differential concentration of progesterone derivatives found in the sciatic nerve between males and females could also contribute to the CIPN sexual dimorphism (Caruso et al., 2013).

Testosterone, the predominant sexual hormone in men could also modulate CIPN symptoms. In the analysis performed for the review, we could not find any research investigating the effect of testosterone on CIPN. However, its role as a TRPM8 agonist and its possible reduction of TRPV1 expression points towards a role on CIPN sex differences (Asuthkar et al., 2015; Bai et al., 2018).

Noteworthy, toll-like receptors (TLR) such as TLR4 and TLR9, that intervene in the TRP molecular pathways, have also shown sex differences in their signaling (Luo et al., 2019; Sorge et al., 2011). Indeed, several chemotherapeutic agents were capable to act as ligands of TLR4 (Park et al., 2014).

Consequently, a growing body of research is evaluating and developing modulators of thermoTRP channels as drug candidates for minimizing CIPN pain symptoms. There, a special focus has been paid on TRPV1 modulators, with capsaicin patches showing pain relief in CIPN patients (Anand et al., 2019; Filipczak-Bryniarska et al., 2017). In this line, the recent development of soft TRPV1 antagonists mitigated paclitaxel-induced sensory symptoms with a safer pharmacological profile (IASP World Congress on Pain 2022, poster n° PFR325: A topical formulation of a soft TRPV1 antagonist (AG1549) alleviates chemotherapy-induced peripheral neuropathy symptoms). In addition to TRPV1, TRPM8 modulators are being tested in different chemotherapeutic regimens. Menthol reduced neuropathic pain scores in CIPN patients and currently, riluzole, an inhibitor of TRPM8 overexpression, is being tested in clinical trials against the sensory symptoms produced under oxaliplatin treatment (Fallon et al., 2015; Kerckhove et al., 2019).

Collectively, inclusion and consideration of both sexes in clinical and preclinical studies would be essential to design more effective and personalized therapies to reduce chronic pain. As a result of the review, we highlighted the relevance of thermoTRP ion channels in migraine and CIPN sexual dimorphism, suggesting them as promising therapeutic targets for a satisfactory management of the pain symptoms in male and female sexes.

Discussion

Discussion

This doctoral thesis has been composed of two main research projects that were based on the development and investigation of paclitaxel and oxaliplatin CIPN models, and one review article for analyzing the advances in pain sexual dimorphism and the implication of TRP channels. Here, I discuss the main results obtained, some limitations of these studies, the relevance and implications of these findings, and the possible future directions in pain research that could be extracted from the learning process that culminates with this thesis defense.

Discussion from Publication 1

In the first project, we investigated the direct effect of **paclitaxel** on the electrogenicity of the DRG sensory neurons of male and female rats. Paclitaxel sensitizing effect peaked 48 h after finishing the treatment, virtually dissipating at 96 h post-treatment. This nociceptor sensitization followed the same kinetics as the pain symptoms developed in patients after paclitaxel treatment, supporting the translational potential of our *in vitro* model (Loprinzi et al., 2011). Indeed, in our long-term cultures we could reproduce previous findings obtained from animal studies such as the increased TRPV1 activity and nociceptors hyperexcitability (Li et al., 2015; Zhang & Dougherty, 2014). Our results also indicate that paclitaxel-induced nociceptor hyperexcitability does not require the immune system intervention, although autocrine action of IL6 and CCK2 could contribute to it (Miller et al., 2009).

Then, we found that paclitaxel was affecting the two distinct populations of small DRG neurons studied, indicating that the altered sensory symptoms could be mediated by both **IB4(-) and IB4(+)** small neurons. However, distinct molecular mechanisms in each population seem responsible of these changes, since TRP channels were only upregulated in IB4(-) neurons (Villalba-Riquelme et al., 2022).

Analysis of the action potential parameters 48 h after paclitaxel treatment resulted in a smaller hyperpolarization phase of the action potential in IB4(-) and IB4(+) neurons. This hyperpolarization phase can be shaped by HCN, K_2P and K_{ir} ion channels. HCN channels open at voltages near the resting membrane potential, allowing the influx of Na^+ and K^+ that opposes to the hyperpolarization voltage decrease and promoting repetitive firing (Benarroch, 2013; Emery et al., 2012; Hogan & Poroli, 2008). Thus, upregulation of the neuronal HCN1 and/or HCN2 ion channels could explain the lower hyperpolarization observed after paclitaxel. In favor of this hypotheses, increased HCN1 mRNA expression was reported in adult rats treated with paclitaxel (Zhang & Dougherty, 2014).

As essential molecular determinants of nociceptors excitability, the activity of the sodium channels **$Na_V1.7$** and **$Na_V1.8$** was investigated. Previous studies indicated that paclitaxel could upregulate the activity of the $Na_V1.7$ channel (Akin et al., 2021; Y. Li et al., 2018; Xia et al., 2016). $Na_V1.7$ activity has been attributed to setting the AP threshold and the current rheobase in nociceptors (Bennett et al., 2019). Other studies, on the contrary, suggested that $Na_V1.8$ was essential in producing the paclitaxel sensitizing effect, since A-803457 and puerarin, antagonists of this channel, could block the paclitaxel-induced hyperexcitability and neuropathic pain, respectively (Verma et al., 2020; Zhang et al., 2018). $Na_V1.8$ channels would contribute to the upstroke phase of the AP, shaping its width, allowing a fast recovery from inactivation, and forming the sodium resurgent currents (Renganathan et al., 2001; Xiao et al., 2019). Thus, $Na_V1.8$ enhanced activity could favor the repetitive AP firing and be implicated in the transition from phasic to tonic firing. When this topic was discussed in the IASP 2022 World Congress on Pain, the possibility that increased $Na_V1.7$ activity could occur on medium-size DRG neurons, whereas $Na_V1.8$ could be altered on small-size DRG neurons arose. This hypothesis would be in accordance with our results, where we could find that paclitaxel increased the activity of $Na_V1.8$ ion channel. Thus, our results points to a major role of $Na_V1.8$ ion channel on paclitaxel hyperexcitability.

Another salient finding of this study was the alteration of the **K_V** current. The reduced fast-inactivating (K_A) current found at depolarizing potentials closed to the AP voltage threshold in IB4(+) neurons (from -40 to 10 mV), might influence the interspike interval favoring a higher firing frequency under stimulation. Meanwhile, the increased K_A current found at depolarizing potentials in IB4(-) neurons might contribute to the faster repolarization phase of the AP, as a larger outflux of K^+ current after the AP overshoot could reduce the voltage faster, approaching the RMP. Due to the huge number of K_V channels and the lack of specific blockers, we could not determine the specific channel subtype involved. Within the K_A channels, the DRG neuron mainly expresses $K_V1.4$, $K_V3.4$, $K_V4.1$ and $K_V4.3$ (Zemel et al., 2018). $K_V1.4$, $K_V4.1$ and $K_V4.3$ are low voltage activated channels that could regulate AP firing and interspike interval, meanwhile $K_V3.4$ is a high voltage activated channel that has been proposed to regulate the repolarization phase of the action potential (Zemel et al., 2018). According to these characteristics, the higher K_A current together with the faster repolarization of the AP exhibited in IB4(-) after paclitaxel treatment, could be the result of an upregulation of $K_V3.4$ activity. On the other side, the reduced K_A current and increased AP firing in IB4(+) following paclitaxel exposure, could be influenced by downregulation in $K_V1.4$, $K_V4.1$ and/or $K_V4.3$. Additional studies would be needed to confirm these hypotheses.

From the thermoTRP channels investigated, we found that paclitaxel was able to increase the activity of **TRPV1** and **TRPM8** specifically in IB4(-) neurons. TRPV1 enhanced activity could explain the

heat hyperalgesia and burning pain experienced by paclitaxel patients (Rowinsky et al., 1993). In accordance with our results, TRPV1 alteration after paclitaxel treatment has been described in numerous preclinical studies (Hara et al., 2013; Li et al., 2015). Therefore, all this information indicates that TRPV1 could be a key therapeutic target for treating paclitaxel pain symptoms. Furthermore, TRPV1 has also shown a crucial role for cancer -induced thermal hyperalgesia in animal models (Ye et al., 2014). Modulating this channel would have a potential role in CIPN and cancer pain on the patients. In addition, our results suggest that TRPM8 upregulation could be the responsible of the cold allodynia symptoms. This data is in agreement with previous studies showing that the specific TRPM8 blocker, AMTB, alleviated the cold hyperalgesia produced in mice treated with paclitaxel (Sałat & Filipek, 2015). Nevertheless, these findings contrast with other studies that proposed that paclitaxel-induced cold hyperalgesia could be the result of TRPA1 upregulation (Materazzi et al., 2012). Intriguingly, we could not find any alteration on TRPA1 activity following paclitaxel treatment. Differences in the CIPN models used could explain these results, since TRPA1 *in vivo* could be upregulated by algesic molecules released by the immune system (Materazzi et al., 2012; Viana, 2016).

Our finding of a more prominent increased sensitization of female nociceptors after paclitaxel treatment is in agreement with the results obtained in recent clinical and preclinical research that indicate a stronger pain sensitivity, incidence and severity in women and female animals, as analyzed in Publication 2 (Cabañero et al., 2022). Previous research attributed the **sex differences** to the effect of sexual hormones on TRP channels signaling (Luo et al., 2021). However, in our results, sex differences in the response to paclitaxel were still present in the DRG cell cultures (Villalba-Riquelme et al., 2022). Here, the use of the same culturing media for both sexes, limits the potential impact that could be produced by fluctuations and different levels of sexual hormones, as occur in animals and humans, suggesting the contribution of additional mechanisms to paclitaxel-induced neuropathy sexual dimorphism. In this line, it is true that differences in the molecular pain pathways, genetics, epigenetics, and protein expression have also been found between male and female subjects (Grant et al., 2022; Mecklenburg et al., 2020; Stephens et al., 2019). Thus, the sexual dimorphism observed could be the result of differences in some of these mechanisms. Also, distinct response of glial cells in each sex could be impacting these results. Hence, future research on the sex differences in pain signaling and the effect of the different sex hormones on the paclitaxel sensitization evoked in cultured nociceptors would provide valuable information on its contribution to PIPN sexual dimorphism. Here, we provide information that suggests that additional factors in nociceptors and/or glial cells could be contributing to paclitaxel CIPN sexual dimorphism.

Sex differences on TRPM8 activity have also been found in our lab in a migraine model, although the presence of testosterone in males was related to its higher functionality. In this case, TRPM8

current density and immunoreactivity was bigger in neurons from male rats, whereas TRPV1 levels remained constant in both sexes. In previous studies, TRPM8 stimulation was shown to inhibit TRPA1 and TRPV1 related migraine pain (Andersen et al., 2016). Thus, one possible implication of this result could be a reduced heat hyperalgesia in males due to the higher TRPM8 functionality. Another possible consequence could be stronger cold hyperalgesia in males following paclitaxel treatment.

Discussion from Oxaliplatin model

As a second step of this doctoral thesis, we investigated the direct effect of **oxaliplatin** on the long-term DRG neuronal cultures from male and female rats. In this case, the major sensitizing effect appeared immediately after the 48 h exposure (0 h post-treatment). Interestingly, this effect was mainly produced by the alteration of the **IB4(+)** neuronal subtype. There, alterations of the passive membrane properties such as the spontaneous activity (SA) and capacitance were found. The higher SA could explain the spontaneous pain experienced by the patients treated with the chemotherapeutic. Meanwhile, the lower capacitance could favor a faster action potential conduction or a more depolarized RMP (Golowasch & Nadim, 2013; Shapiro et al., 2012). In addition, the tendency for a higher input resistance observed in IB4(+) neurons could contribute to the lower rheobase detected after oxaliplatin treatment, since the current injected would produce larger voltage changes, facilitating reaching the voltage threshold for AP firing.

In response to electrical stimulation, the hyperexcitability of IB4(+) neurons was marked by a lower rheobase and a higher firing frequency developed 0 h post-treatment with oxaliplatin. The changes that we found on the **AP parameters** of IB4(+) neurons consisted in an augment in the maximum voltage reached in the depolarization (overshoot), amplitude and the time necessary to reach the depolarization peak. The depolarization phase is produced by the influx of Na^+ ions inside the cell through Na_V channels, and therefore increased functionality of these channels could favor a more pronounced entry of Na^+ inside the cell, increasing the AP overshoot and amplitude.

For IB4(-), although no significant differences were found in the RMP, rheobase or firing frequency, the percentage of neurons with spontaneous activity significantly augmented 48 h and 96 h post-treatment with oxaliplatin. Thus, we cannot discard a minor contribution of IB4(-) neurons to the symptoms associated with the oxaliplatin-induced neuropathy.

At the peak of excitability of IB4(+) neurons, we examined the functionality of the ion channels implicated in the neuronal electrogenicity to elucidate the potential underlying mechanisms. First, we

investigated the **Na_V** channels activity, but the current density did not seem to be affected after oxaliplatin exposure. However, these currents exhibited a slower inactivation kinetics, resulting in higher time of the channel remaining opened. The slower inactivation could explain the bigger overshoot and AP amplitude observed, since inactivation of the Na_V channels stops the Na^+ influx limiting the membrane depolarization. Moreover, faster recovery from inactivation was produced in the oxaliplatin group. Na_V inactivation is a key mechanism that arises after an action potential is fired to limit the following AP that could be fired during the refractory period. Thus, both observations could contribute to the higher firing frequency of the neurons detected.

Na_V functionality in oxaliplatin models has also been investigated in the literature. Detection of a $Na_V1.6$ persistent and resurgent current was reported after oxaliplatin treatment, but this effect was restricted to large diameter DRG neurons and was absent in small neurons (Sittl et al., 2012). Similarly, we could not find $Na_V1.6$ differences in small IB4(+) DRG neurons. Another study described upregulation of $Na_V1.8$ mRNA in response to oxaliplatin and its blockage with dexpramipexole provided analgesia in mice treated with this chemotherapeutic (Descoeur et al., 2011; Urru et al., 2020). There, we observed a tendency to increase $Na_V1.8$ current density and slower inactivation kinetics although no statistical significance was reached. Currently, no studies have linked $Na_V1.7$ alterations to oxaliplatin neuropathy. Thus, between Na_V channels, $Na_V1.6$ and $Na_V1.8$ appear to have a major potential involvement in oxaliplatin neuropathy.

As crucial determinants of the electrogenicity of the neurons, **K_V** channels activity was investigated after vehicle and oxaliplatin treatment. However, no differences were detected in the current density, although a shift of the G-V curve of K_A current to more positive potentials could be distinguished. To date, poor information on K_V modulation by oxaliplatin has been published. Descoeur et al. (2011) reported downregulation of $K_V1.1$ ion channel following oxaliplatin injury. In trigeminal ganglia, downregulation of the delayed-rectifying $K_V7.2$ by oxaliplatin has also been described but additional studies are needed to test if this mechanism is shared by DRG neurons (Ling et al., 2017).

Among the noxious sensors, TRP ion channels appear essential for the oxaliplatin-induced neuropathic pain symptoms. In our work, IB4(+) neurons exhibited increased **TRPA1** and **TRPV1** current responses, suggesting the relevance of these channels in oxaliplatin neuropathy. This was further corroborated using the MEA technique, where the electrical firing in response to specific agonists of TRPA1 and TRPV1 significantly increased. Similar to our results, in a rat model of oxaliplatin-induced neuropathy higher expression of TRPA1 and TRPV1 was found in small IB4(+) DRG neurons after treatment, supporting these results and the translational potential of the oxaliplatin model (Chukyo et al., 2018; Nassini et al., 2011). Higher TRPA1 and TRPV1 responses were also observed after

a 48 h oxaliplatin treatment by Anand et al. (2010). In contrast, the work from Wu et al. (2021) attributed the oxaliplatin neuropathy symptoms to the depolarization of IB4(-) neurons and TRPM8 increased activity without altering IB4(+) neurons. These differences seem to account for the short (15-60 min) incubation of oxaliplatin that was performed in that study that was more similar to the acute allodynia evoked by oxaliplatin during its infusion before longer structural changes could be developed (Ventzel et al., 2016). In agreement with this hypothesis, Rimola et al. (2021) showed that the changes evoked in TRPM8 were transient, showing increased activity at 1 h but decreased levels at 24 h after treatment. Indeed, other studies investigating longer time exposures of oxaliplatin did not detect higher TRPM8 activity in small DRG neurons (Chukyo et al., 2018; Descoeur et al., 2011).

As a potential molecular mechanism explaining the potentiation on TRPV1 and TRPA1 functionality, we detected upregulation of their respective mRNAs at the peak of their activity. Higher TRPA1 mRNA levels were also previously detected and localized in small DRG neurons of mice treated with oxaliplatin (Descoeur et al., 2011; Yamamoto et al., 2015). In addition, in a recent study, authors found increased TRPV1, TRPA1 and TRPM8 mRNA levels in rat after multiple oxaliplatin applications (Miguel et al., 2022). Altogether, upregulation of TRPV1 and TRPA1 ion channels seem to have a substantial contribution to OIPN pathophysiology.

Other ion channels that have been shown to be altered by oxaliplatin are **TREK**, **TRAAK** and **HCN** channels (Descoeur et al., 2011; Dionisi et al., 2020). Descoeur et al. (2011) detected TREK1 and TRAAK downregulation and upregulation of HCN1 mRNA. These alterations could result in reduced K^+ background current, thus depolarizing the resting membrane potential or reducing the AP hyperpolarization phase. Indeed, blockage of HCN1 has already been considered as a possible strategy to diminish oxaliplatin neuropathy symptoms (Resta et al., 2018). Novel research is also pointing that inhibition of epigenetic co-repressors histones deacetylases prevented K_2P downregulation reducing oxaliplatin-induced acute cold and mechanical hypersensitivity (Pereira et al., 2021). In our work, we could not investigate the functionality of these channels, but their alterations could also contribute to the increased excitability and in the depolarized RMP of female neurons. Further research would be needed to establish the contribution of these channels to oxaliplatin CIPN.

Another salient finding of our work is the **sexual dimorphism** observed in the excitability and molecular mechanisms altered after oxaliplatin treatment. Recent research has reported sex differences in OIPN, showing females more severe cold allodynia (Miguel et al., 2022). However, poor information regarding the potential differences in the function and molecular mechanisms has been published. In our research, first, depolarization of the RMP was observed only in female cultures following oxaliplatin exposure. Since the RMP is mainly set by the activity of K^+ channels, K_V and K_2P

ion channels could be implicated. Investigation of K_V ion channels showed a reduction in the activity of fast-inactivating K_A at voltages near -10 mV and a shift in the GV curve to more positive potentials. However, this effect occurring at higher voltages than the RMP might contribute to the higher AP amplitude, to reduce the interspike interval and/or favoring the AP tonic firing rather than depolarizing the RMP. This hyperexcitability in females could also be favored by the enhanced Na_V current density observed at V ≥ -40 mV, since it could reduce the rheobase and promote repetitive AP firing. In contrast, male treated cultures exhibited similar K_V currents, but showed significant alterations in the Na_V inactivation and voltage dependence. There, a faster recovery from inactivation and slower inactivation kinetics was detected only in this sex, where it could also favor a higher firing frequency. Additional experiments would be needed to clarify the exact impact of these molecular differences on male and female OIPN.

Then, we investigated sex differences on TRP channels. Immediately after oxaliplatin treatment, the percentage of neurons with response to AITC, the TRPA1 agonist, was significantly higher only in male neurons. However, the TRPA1 sex difference seemed to be restored at 48 h after treatment when all the neurons registered in the treated group responded to AITC. The reason behind the differences in the TRPA1 sensitization time course remains to be elucidated. Differences in the TRPA1 faster upregulation mechanisms might slower its higher activity in females following oxaliplatin treatment.

As a result, we developed two ***in vitro* preclinical models** that could reproduce the neuropathy produced by paclitaxel and oxaliplatin. A limitation of our studies is that we started investigating the action of an individual chemotherapeutic drug (paclitaxel and oxaliplatin), but in the clinical practice, cancer patients usually receive a combination of chemotherapeutic drugs (Table 2) (Cabañero et al., 2022). As a future interesting approach, exposure of the same long-term cultures to several chemotherapeutic drugs could also provide very interesting information on how the neurons respond when the different actions concur, since synergic or antagonistic mechanisms might cause different reactions.

Besides, some limitations from the culture origin might be noted. We have developed these models from **rat DRG neurons**, that are closer than the mice neurons to humans and are preferred in pharmacological studies for the translational potential of the results (Prior et al., 2020). However, the translational potential of these models could be increased using human DRG neurons or human induced pluripotent stem cells (iPSC). Human DRG neurons are very scarce and, as a flaw of the iPSC, the low rate of successful conversion achieved and the inability to induce all the heterogeneity of neurons subtypes found in the DRG, complicated its use. In any case, our *in vitro* models also pave the

way for the use of human neuronal cultures, since we have stablished a translational protocol that could be extrapolated to other *in vitro* cultures.

For analysing the functional and molecular changes evoked in these models, we used a wide variety of laboratory techniques such as **patch clamp**, **MEA**, **cell culture**, **qPCR**, **Western blot**, and **Immunocytochemistry**. Nonetheless, the use of additional techniques such as single-cell RT-PCR could also provide an interesting correlate between the molecular and functional changes observed in each cell.

In comparison to **previous *in vitro* studies** done incubating paclitaxel and oxaliplatin, we prolonged the culturing time since most of them exposed the DRG cultures 1-2 days after cell seeding (Dionisi et al., 2020; Marcotti et al., 2023). This avoids the recovery of the axonal transport dependent mechanisms, thus interfering with the analysis of the complete changes evoked on mature DRG neurons. As an important advantage, our study faces this problem by waiting 4-5 days before the treatment to allow the axonal sprouting of the neurons and *de novo* formation of microtubules. As a novel approach, we also investigated the time course of the chemotherapeutic neuropathy by performing the experiments at 0 h, 48 h, and 96 h after finishing the treatment, allowing us to analyze the sensitization and desensitization processes evoked.

Altogether, our *in vitro* pre-clinical models of paclitaxel- and oxaliplatin-induced peripheral neuropathy provide a powerful tool for investigating the molecular mechanisms underlying peripheral neuropathies, identifying therapeutic targets for managing the pain symptoms and for testing potential analgesic compounds.

Discussion from Publication 2

Due to the emerging evidence of the presence of sexual dimorphism on different pain disorders, we analyzed this topic elaborating a review article. There, we focused on **migraine** and **CIPN** as two pain syndromes with a strong peripheral component. As stated in the review, sex differences have also been reported in other chronic pain disorders. Among them, we can find osteoarthritis, rheumatoid arthritis, fibromyalgia, low back pain, irritable bowel syndrome, or peripheral diabetic neuropathy (Giatti et al., 2019; Kovacs & Olsen, 2011; Lee et al., 2015; Mas et al., 2008; Plotnikoff et al., 2015; Voß et al., 2012). Thus, future research addressing the presence of sexual dimorphism in their physiopathological pathways would be necessary.

In **migraine**, sex has been considered a risk factor with 35 % of women and 15 % of men experiencing this disorder (Stovner, 2018). As a possible explanatory mechanism, we highlighted the sex hormones related modulation of TRP channels. There, we focused on examining the role of TRPA1, TRPV1 and TRPM8 ion channels due to the higher evidence endorsing its crucial participation initiating migraine attacks (Alarcón-Alarcón et al., 2022; Benemei & Dussor, 2019; Yakubova et al., 2021). Hormonal modulation of these TRPs by estrogens, testosterone, prolactin, or progesterone were suggested as sources of migraine sex differences. Nonetheless, apart from these channels, we also indicated the potential involvement of other thermoTRPs such as TRPV3, TRPV4, and TRPM3, suggesting the need of future research examining their role in migraine pathophysiology.

For **CIPN**, sex differences have been largely overlooked due to its complex etiology. The coexistence of numerous interfering factors in the patients such as tumor metabolism, surgical trauma, immune responses, radiotherapy and/or anti-hormonal therapies, the use of several co-administered drugs, and the differences in their pharmacokinetics and pharmacodynamics profiles, difficulted its analysis in clinical studies (Wagner, 2020). In addition, most of the research has been performed in male subjects and the possible existence of sex differences has been ignored. However, novel research is starting to address this issue, resulting in the discovery of sex differences in the pain symptoms and physiopathological pathways of CIPN. Among the most common clinical findings, CIPN incidence, sensitivity and/or severity was higher in women for most of the chemotherapeutic regimens (Table 1, Publication 2). Thus, one of the conclusions extracted in our article is the urgent need that clinical and pre-clinical studies evaluate CIPN in male and female subjects.

There, it is well-known that **TRP channels** alterations are essential in migraine and CIPN pain symptoms (Alarcón-Alarcón et al., 2022; Villalba-Riquelme et al., 2022). In addition, sex differences on TRP channel signaling have increasingly been reported (Asuthkar et al., 2015; Luo et al., 2021). Indeed, evaluation of potential sexual dimorphic mechanisms of migraine and CIPN also showed a crucial role for these channels on their pathophysiology. As a major mechanism, the TRP signaling modulation by sexual hormones appears as a key molecular mechanism underlying migraine and CIPN sexual dimorphism, with estrogens showing a pronociceptive effect, whereas progesterone and testosterone seemed to protect against the pain symptoms (Alarcón-Alarcón et al., 2022).

Consequently, several studies are evaluating thermoTRP channel modulators as a promising strategy for alleviating CIPN (Anand et al., 2019; Fallon et al., 2015; Filipczak-Bryniarska et al., 2017; Kerckhove et al., 2019; Privitera & Anand, 2021). There, for chronic pain with a strong peripheral component, we remarked as a recent interesting **therapeutic approach** the use of topical ointments

formulations that could reduce the pain symptoms with unsignificant adverse effects due to the avoidance of systemic distribution (Serafini et al., 2018).

In the review, we also detected some gaps in the research of sexual dimorphism on chronic pain. For instance, we could not find articles that explored the contribution of testosterone to CIPN. Due to the recent discovery of its implication in the sexual dimorphism in other chronic pain conditions (Alarcón-Alarcón et al., 2022), **future research** would be needed to address this topic. Blocking the testosterone receptor with specific antagonists or exposing the *in vitro* CIPN models to the presence of this hormone could give us a hint on its specific role on CIPN pain pathways.

Conclusiones/Conclusions

Conclusiones

Considerando los objetivos iniciales y contrastándolos con los resultados obtenidos en nuestro trabajo, se han extraído las siguientes conclusiones:

En base al trabajo realizado en la **Publicación 1**, ***Paclitaxel in vitro reversibly sensitizes the excitability of IB4(-) and IB4(+) sensory neurons from male and female rats***, se puede concluir:

1. Paclitaxel aumentó la excitabilidad de las neuronas IB4(-) e IB4(+) de forma directa y reversible.
2. Las actividades de los canales $Na_V1.8$, TRPV1 y TRPM8 se potenciaron tras el tratamiento con paclitaxel, emergiendo como dianas terapéuticas clave para tratar los síntomas de dolor derivados de la neuropatía inducida por paclitaxel.
3. La sensibilización producida por paclitaxel estuvo marcada por la presencia de dimorfismo sexual, mostrándose los nociceptores de ratas hembra más sensibles al aumento de excitabilidad.
4. Nuestro modelo preclínico *in vitro* de la neuropatía inducida por paclitaxel proporciona una herramienta poderosa para investigar los mecanismos moleculares subyacentes, identificar potenciales dianas terapéuticas y testar compuestos analgésicos para hacer frente a los síntomas de dolor.

En base a los resultados obtenidos con el **modelo con Oxaliplatino**, puede concluirse:

1. Oxaliplatino sensibilizó de forma directa y reversible la actividad de las neuronas IB4(+).
2. La actividad de TRPV1 y TRPA1 se incrementó, mientras que los canales Na_V mostraron una cinética de la inactivación más lenta después de la exposición a oxaliplatino, indicando su papel como potenciales dianas terapéuticas para tratar los síntomas de dolor producidos por dicha neuropatía.
3. La sensibilización por oxaliplatino exhibió un dimorfismo sexual en el que las neuronas de ratas hembra mostraron un potencial de membrana más despolarizado y menor actividad de los K_V, mientras que en las de los machos se observó un aumento más pronunciado en la actividad de TRPA1 y una inactivación enlentecida de los Na_V.

De acuerdo al trabajo realizado en la **Publicación 2**, ***ThermoTRP channels in pain sexual dimorphism: new insights for drug intervention***, se puede concluir:

1. Evidencias crecientes indican la presencia de dimorfismo sexual en la sintomatología y fisiopatología de la migraña y CIPN.
2. Los canales TRP constituyen dianas terapéuticas clave para el tratamiento de la migraña y la CIPN en ambos sexos.
3. Los estudios clínicos y preclínicos deberían ser sensibles al sexo de los individuos para poder alcanzar un manejo satisfactorio del dolor en toda la población.

Conclusions

Regarding the work from **Publication 1**, ***Paclitaxel in vitro reversibly sensitizes the excitability of IB4(-) and IB4(+) sensory neurons from male and female rats***, it can be concluded that:

1. Paclitaxel directly and reversibly increased the excitability of IB4(-) and IB4(+) neurons.
2. $Na_V1.8$, TRPV1 and TRPM8 activities were enhanced after paclitaxel treatment, emerging as key therapeutic targets for treating the pain symptoms derived from paclitaxel-induced peripheral neuropathy.
3. Paclitaxel sensitization exhibited a sexual dimorphic response with nociceptors from female rats being more sensitive to the treatment.
4. Our *in vitro* pre-clinical model of paclitaxel-induced peripheral neuropathy provides a powerful tool for investigating the molecular mechanisms underlying the peripheral neuropathy, identifying potential therapeutic for pain symptoms and for testing analgesic compounds.

Regarding the work from **Oxaliplatin model**, it can be concluded that:

1. Oxaliplatin directly and reversibly sensitized the activity of IB4(+) neurons.
2. After oxaliplatin treatment, TRPV1 and TRPA1 were upregulated, whereas Na_V channels exhibited a slower inactivation kinetics, indicating these channels as potential therapeutic targets for treating the consequent pain symptoms.
3. Oxaliplatin sensitization showed a sexual dimorphism with females showing a more depolarized resting membrane potential and decreased K_V activity, meanwhile males exhibited a more pronounced increase in TRPA1 activity and slower Na_V inactivation.

Regarding the work from **Publication 2**, ***ThermoTRP channels in pain sexual dimorphism: new insights for drug intervention***, it can be concluded that:

1. Emerging evidence indicates the presence of sexual dimorphism in migraine and CIPN.
2. TRP channels are key therapeutic targets for treating migraine and CIPN in males and females.
3. Clinical and pre-clinical studies should be sensitive to sex to reach a satisfactory pain management in all individuals.

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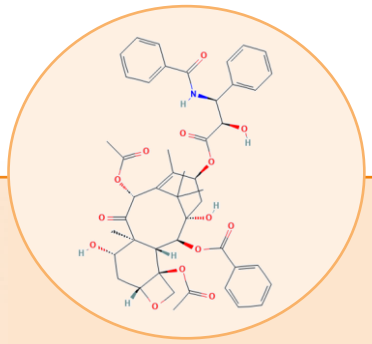
Annexes

Annex 1.
Publication 1

BJP British Journal of Pharmacology



Paclitaxel in vitro reversibly sensitizes the excitability of IB4(-) and IB4(+) sensory neurons from male and female rats

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RESEARCH ARTICLE

Paclitaxel in vitro reversibly sensitizes the excitability of IB4(−) and IB4(+) sensory neurons from male and female rats

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Background and Purpose: Paclitaxel produces a chemotherapy-induced peripheral neuropathy that persists in 50–60% of cancer patients upon treatment. Evidence from animal models suggests an axonopathy of peripheral A- and C-type fibres that affects their excitability. However, direct effects of paclitaxel on sensory neuron excitability and sexual dimorphism remain poorly understood.

Experimental Approach: We used a long-lasting (10 days in vitro) primary culture of rat dorsal root ganglion (DRG) neurons to investigate the time course effect of paclitaxel on the electrical activity of IB4(−) and IB4(+) sensory neurons of female and male adult Wistar rats.

Key Results: Paclitaxel strongly and reversibly stimulated spontaneous activity and augmented action potential tonic firing in IB4(−) and IB4(+) neurons in both sexes, peaking at 48 h post-treatment and virtually disappearing at 96 h. Paclitaxel decreased the current rheobase for action potential firing by reducing and accelerating the after-hyperpolarization phase. Molecularly, paclitaxel modulated Na^+ and K^+ ion currents. Particularly, the drug significantly augmented the function of $Na_v1.8$, TRPV1 and TRPM8 channels. Furthermore, paclitaxel increased $Na_v1.8$ and TRPV1 expression at 48 h post-treatment. Notably, we observed that female DRG neurons appear more sensitive to paclitaxel sensitization than their male counterparts.

Conclusions and Implications: Our data indicate that paclitaxel similarly potentiated IB4(−) and IB4(+) electrogenicity and uncover a potential sex dimorphism in paclitaxel-induced chemotherapy-induced peripheral neuropathy. Our in vitro, preclinical, chemotherapy-induced peripheral neuropathy paradigm provides a tool for studying the dynamics and underlying molecular mechanisms contributing to nociceptor sensitization in peripheral neuropathies and for testing desensitizing compounds.

Abbreviations: AHP, after-hyperpolarization phase; AITC, allyl isothiocyanate; AP, action potential; CIPN, chemotherapy induced peripheral neuropathy; DIV, days in vitro; DRG, dorsal root ganglion; G-V, conductance–voltage relationship; IB4, isolectin B4; iPSC, induced pluripotent stem cells; J-V, Current density–voltage relationship; K_A, fast-inactivating potassium current; K_{DR}, delayed-rectifier potassium current; MEA, microelectrode array; RMP, resting membrane potential; Rpl29, ribosomal protein L29; SA, spontaneous activity; t_{AHP}, time to recover from the AHP; t_{peak}, time to reach the maximum amplitude; t_r, repolarization time; TTX, tetrodotoxin; V_{th}, voltage threshold.

KEYWORDS
cancer, ion channel, neuropathy, nociceptor, pain, sexual dimorphism, thermoTRP

1 | INTRODUCTION

Paclitaxel is used as a first-line chemotherapeutic drug to treat several solid tumours (Abu Samaan et al., 2019; Markman, 1991;). This drug also induces a peripheral neuropathy (chemotherapy-induced peripheral neuropathy, CIPN), with a prevalence of up to 90% of patients during treatment that may persist for up to 48 months post-treatment (Colvin, 2019; Seretny et al., 2014). CIPN is a sensory disorder characterized by both paraesthesia and dysesthesia, encompassing numbness, tingling, shooting, burning, stabbing and throbbing pain and frequently dysfunctional motor function (da Costa et al., 2020; Tanay et al., 2017). These sensory symptoms point to a central role of the epidermal nociceptive system in CIPN pathophysiology (Zajączkowska et al., 2019).

Paclitaxel stabilizes microtubules,thus inhibiting mitosis in proliferating cells (Xiao et al., 2006). In nociceptive neurons, however, interference with microtubule dynamics inhibits axonal transport affecting epidermal sensory fibre innervation (Smith et al., 2016; Zajączkowska et al., 2019). Thus, paclitaxel produces an axonopathy that appears to underlie the sensory alterations suffered by cancer patients receiving the drug, including an increase of the electrical excitability of sensory fibres (Zhang & Dougherty, 2014). Higher neural excitability arises from an increase in the expression of **voltage-gated Na^+** and **Ca^{2+} channels** and a decrease of **voltage-gated K^+ ion channels** (Waxman & Zamponi, 2014). **TRP channels**, mitochondrial dysfunction and oxidative stress have been also involved (Zajączkowska et al., 2019). Analytical studies of CIPN have used primary cultures of nociceptors obtained from rodents treated with paclitaxel following a range of regimes, from acute to chronic, to reproduce the thermal and mechanical hypersensitivity seen in humans (Hara et al., 2013; Smith et al., 2004). These studies suggested that paclitaxel also promoted the release of cytokines and chemokines from immune cells (Doyle et al., 2012; Son et al., 2019) that, in turn, sensitized nociceptors contributing to the CIPN sensory abnormalities (Zajączkowska et al., 2019). Thus, the direct effect of paclitaxel on sensory neurons excitability remains poorly understood, as previous studies involved multifactorial components arising from the action of paclitaxel in different types of cells.

Primary cultures of DRG neurons have been proposed to preclinically model CIPN in vitro (Eldridge et al., 2020; Malgrange et al., 1994; Scuteri et al., 2006). Li et al. reported an in vitro model of nociceptors in culture that faithfully reproduced the acute toxicity of paclitaxel, including an increase in p**ERK** and p**p38** expression along with an increment in the expression of algesic factors that resulted in an increase in the excitability of nociceptors (Li et al., 2020). In our present experiments, we have used a long-term, in vitro culture of DRG neurons (10 DIV) to investigate the time course of paclitaxel-induced sensitization of neural excitability after drug removal. We analysed the effect of paclitaxel on both IB4(−) and IB4(+) neural subpopulations, as well as in sensory neurons isolated from female and male rats, to evaluate the presence of any sexual dimorphism. We report that paclitaxel reversibly potentiated neural excitability, with a maximum at 48 h after drug treatment that had virtually dissipated 96 h post-treatment. Neural excitability resulted primarily in a decrease of the current rheobase in both neural populations. Molecularly, paclitaxel modulated Na^+ and K^+ currents that define AP waveform generation and the activity of **TRPV1** and **TRPM8** channels. Notably, we observed that DRG neurons from female rats seemed to be more sensitive to paclitaxel-evoked excitability than those derived from male rats. Taken together, our findings indicate a direct and reversible effect of paclitaxel on the excitability of IB4(−) and IB4(+) and a potential sex-related dimorphism on sensory neurons.

What is already known

- Paclitaxel produces an axonopathy that appears to underlie the sensory alterations in chemotherapy-induced peripheral neuropathy.
- Paclitaxel promotes the release of cytokines and chemokines from immune cells that sensitize sensory neurons.

What this study adds

- Paclitaxel directly and reversibly increases IB4(−) and IB4(+) nociceptor excitability.
- Paclitaxel sensitization exhibits sex dimorphism as nociceptors from female rats are more sensitive.

What is the clinical significance

- $Na_v1.8$ and TRPV1 channels are key therapeutic targets for treating chemotherapy-induced peripheral neuropathy.
- Topical TRPV1 antagonists appear to be clinically useful therapeutics for alleviating chemotherapy-induced peripheral neuropathy symptomatology.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures were approved by the Institutional Animal and Ethical Committee of the Miguel Hernández University of Elche (UMH.IDI.AFM.06.20) and the Autonomous Government of Valencia (2021/VSC/PEA/0089), in accordance with the guidelines of the Economic European Community in accordance to Directive 2010/63/EU, the National Institutes of Health, and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Most of the in vivo studies regarding the neurotoxic effects of paclitaxel have been previously performed on Wistar rats due to the similarities with the human pain pathway (Hara et al., 2013; Li et al., 2018). Therefore, our experiments were conducted on 12–20 weeks old male and female Wistar rats obtained from Servicio de Experimentación Animal (SEA) of Miguel Hernández University of Elche. Animals were housed in polycarbonate plastic cages (two to four animals per cage) at 21–23°C with a 12,h light/dark cycle in a controlled environment with water and food available ad libitum.

2.2 | Culture of DRG neurons

Rats under isoflurane anaesthesia (IsoFlo®, Zoetis) were decapitated, and the vertebral column was excised. DRG were then isolated in a Petri dish containing Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) with the use of a stereo microscope (VWR). Thereafter, DRG neurons were isolated by incubating the ganglia with 0.25% (w/v) collagenase type IA (Sigma-Aldrich) in DMEM (Gibco) with 1% penicillin–streptomycin (P/S, 5000 U·ml^{-1}, Invitrogen) at 37°C for 1 h in 5% CO_2. After digestion, the DRG was mechanically dissociated by pipetting, placed into DMEM medium containing 10% of foetal bovine serum (FBS, Invitrogen) and 1% P/S and centrifuged at 300 x *g* at ≈22ºC for 5 min. The supernatant with containing connective tissue was discarded and the cell pellet was resuspended. This centrifugation and resuspension procedure was performed 3 times. Cell culturing conditions were adapted from a previously described protocol (Newberry et al., 2016). One hour later, cell medium was replaced by Neurobasal-A medium (Gibco) supplemented with 1% P/S, B-27® Supplement (2%; Gibco) and GlutaMAX$_{TM}$ (1%; Invitrogen). Nerve growth factor (NGF, 5 ng·ml^{-1}; Sigma-Aldrich), NaCl (5 mg·ml^{-1}; Sigma-Aldrich), uridine (17.5 μg·ml^{-1}; Sigma-Aldrich) and 5-fluoro-2-deoxyuridine (7.5 μg·ml^{-1}; Sigma-Aldrich) were added to the cell medium 24 h after seeding. For microelectrode arrays (MEA), due to technical requirements, the final NGF concentration was 100 ng·ml^{-1}. Cells were maintained at 37°C in a humidified incubator with 5% CO_2. During cell culture, half of the medium was changed for fresh medium every 3–4 days. Five days after cell seeding, cells were treated with 1 μM of paclitaxel or with the vehicle (0.04% DMSO) for 24 h.

2.3 | Immunofluorescence staining

DRG neurons seeded in coverslips were washed with DPBS and fixed in 4% (v/v) paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After three washes with DPBS, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 5 min and blocked with 5% normal goat serum (NGS, Sigma-Aldrich) for 1 h at ≈22°C. Then, cells were incubated overnight at 4°C in DPBS solution with 5% NGS containing primary antibodies against the following targets: neuron-specific protein NeuN using a mouse monoclonal antibody at 1:50 dilution (Millipore, Cat# MAB377C3, RRID:AB_10918200); TRPV1 using a rabbit polyclonal antibody at 1:100 dilution (Alomone Labs, Cat# ACC-029, RRID:AB_2040258); **TRPA1**, with a rabbit polyclonal antibody, 1:100 (Alomone Labs, Cat# ACC-037, RRID:AB_2040232); TRPM8, with a rabbit polyclonal antibody, 1:100 (Alomone Labs, Cat# ACC-049, RRID:AB_2040254); **$Na_V1.7$**, with a rabbit polyclonal antibody, 1:200 (Alomone Labs, Cat# ASC-008, RRID:AB_2040198); **$Na_V1.8$**, with a rabbit polyclonal antibody, 1:200 (Alomone Labs, Cat# ASC-016, RRID:AB_2040188); and **$Na_V1.9$**, with a rabbit polyclonal antibody 1:200 (Alomone Labs Cat# ASC-017, RRID:AB_2040200), respectively. The following day, cells were washed with DPBS and incubated with goat anti-rabbit IgG Alexa Fluor® 488, 1:500 (Thermo Fisher Scientific, Cat# A-11034, RRID:AB_2576217) and goat anti-mouse IgG Alexa Fluor® 568, 1:500 (Thermo Fisher Scientific, Cat# A-11031, RRID:AB_144696) secondary antibodies at ≈22°C for 1 h. After DPBS washed, cells were stained with DAPI, 300 nM (Thermo Fisher Scientific Cat# D1306, RRID:AB_2629482). Coverslips were mounted with Mowiol® (Calbiochem). All dilutions were freshly prepared the day of the experiment from original stocks stored in individual aliquots at −20°C. Samples were visualized with an inverted fluorescence microscope (Zeiss Axio Observer, Carl Zeiss). Fluorescence was quantified removing the mean background fluorescence from each cell fluorescence using Fiji® software (Fiji, RRID:SCR_002285) (Schindelin et al., 2012). Representative pictures shown were obtained with an inverted confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss). Experimental details of the immunocytochemical procedures conform with BJP guidelines.

2.4 | Patch-clamp recordings

Small-diameter (<30 μm) DRG neurons seeded in coverslips were registered in voltage and current-clamp modes. For cell culturing, crystals were coated with poly-L-lysine (50 μg·ml^{-1}, Sigma-Aldrich) for 2 h. After four washes with deionized water, crystals were incubated during 1 h at 37°C with laminin diluted in DMEM medium (10 μg·ml^{-1}; Sigma-Aldrich). After DRG neurons extraction,

laminin was replaced by the cell suspension diluted in DMEM 1% FBS 1% P/S. Patch pipettes from borosilicate glass with OD 1.5 mm × ID 1.17 mm (Warner Instruments) were pulled using a Flaming/Brown micropipette puller P-97 (Sutter Instruments) to have 2–5 MΩ resistance. Seal resistance was between 200 MΩ and 1.5 GΩ, and series resistance was compensated around 80%. Extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, 20 mannitol, pH 7.4 adjusted with NaOH. Pipette internal solution contained (in mM): 144 KCl, 2 $MgCl_2$, 10 HEPES, 5 EGTA, pH 7.2 adjusted with KOH. Experiments were performed in whole-cell configuration at ≈22°C. After establishing whole-cell access, cells were recorded in current-clamp mode. First, the resting membrane potential (RMP) was determined without any current injection. Cells with RMP higher than −40 mV or AP that did not overshoot 0 mV were not considered for analysis. Neurons that fired action potentials in the absence of stimulus were considered as having spontaneous activity (SA). To calculate the rheobase, the firing frequency and to classify neurons into tonic or phasic behaviour, 1 s current depolarizing pulses from 0 to 300 pA in 10 pA intervals were applied. The minimum current required to evoke the first action potential was considered as the current rheobase. Neurons were classified as phasic if they fired one or few action potentials at the onset of the current stimulus. Tonic neurons were those that were able to fire continuously during one or more of the 1 s current pulses. The AP parameters were measured in the action potential fired at the minimum current injected using 10 ms depolarizing pulses from 0 to 300 pA in 10 pA steps. AP threshold was considered when the upstroke slope was ≥10 $V{\cdot}s^{-1}$. AP amplitude was measured from RMP to peak.

For voltage-clamp recordings, capacitive transients were compensated. Cells with capacitance values higher than 40 pF were excluded from the analysis. For measuring K^+ currents, the fast-inactivating K^+ current (K_A) and the non-inactivating K^+ current remaining before the end of the protocol (K_{DR}), respectively, were measured in a 300 ms voltage-step protocol from −80 to 70 mV in 10-mV intervals. For registering Na^+ currents, external solution contained (in mM): 70 NaCl, 65 choline chloride, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 TEA-Cl, $CsCl_2$ 10 HEPES and 10 glucose, pH 7.4 adjusted with NaOH. Pipette internal solution contained (in mM): 140 CsF, 10 NaCl, 1 EGTA, 5 glucose and 10 HEPES, pH 7.30 adjusted with CsOH. $Na_V1.8$ currents were isolated using previous described voltage steps protocols (Soriano et al., 2019), The G-V curves were calculated from current–voltage relations, using $G = I_x/(V - V_x)$, where I_x and V_x are the ionic currents and the equilibrium potential for Na^+ or K^+, respectively. These curves were fitted to Boltzmann equation and the voltage for half-maximum activation ($V_{1/2}$) and gating valence (z) were determined (details are given in figure legends).

Current responses to capsaicin, allyl isothiocyanate (AITC), menthol and menthol with AMTB were measured using a continuous protocol at −60 mV. These compounds were applied diluted in external solution using a continuous perfusion system (10 $ml{\cdot}min^{-1}$). To activate TRPV1 and TRPA1 channels, four 1 μM capsaicin pulses of 15 s duration followed by a 60 s pulse of 100-μM AITC were applied, respectively. TRPM8 channel currents were elicited by a 20 s pulse of 100 μM menthol. Then, the TRPM8 channel blocker AMTB was used at 10 μM with menthol for 20 s. Data were acquired at 20 kHz for all the protocols except for the continuous voltage protocols performed to study capsaicin, AITC and menthol responses that were sampled at 1 kHz. These currents were additionally filtered to 2 Hz for plotting. Recordings were performed with an EPC10 amplifier controlled by Patchmaster software (HEKA Elektronik).

To study separately peptidergic and non-peptidergic DRG neurons, before each measurement, coverslips were incubated for 10 min with the fluorescent dye Isolectin-GS-IB4 Alexa Fluor® 568 conjugate (10 $\mu g{\cdot}ml^{-1}$; Invitrogen) diluted in external solution, followed by two washes. As previously described, IB4 labelling did not alter viability or the electrophysiological parameters of the neurons (Stucky & Lewin, 1999). Cells were visualized with a fluorescent microscope (Axiovert 200 Inverted Microscope, Carl Zeiss) with an excitation filter ET545 and an emission filter ET605 (CHR-49004, Laser 2000 SAD). Cells that did not show fluorescence were considered as IB4(−). Only one cell per dish was recorded and analysed.

2.5 | Quantitative RT-PCR

Total RNA was isolated using the E.Z.N.A.® microElute total RNA kit (Omega Bio-tek). Extracellular RNA samples quantity and purity were analysed using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific). Possible contaminating DNA was digested using the DNase I (Sigma), and then the RNA extracted was reverse transcribed using the First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Primers targeting rat selective channels were designed, and their sequences were as follows: TRPV1: Fw: 5′ TGGACGAGGTAAAC TGGACT, Rv: AGTTTCTCCCTGAAACTCGG; TRPA1: Fw: 5′ AGTGGC AATGTGGAGCGATA Rv: 5′ TCCCGTCGATCTCAGCAATG; TRPM8: Fw: 5′ GCTACGGACCAGCATTTCAT, Rv: 5′ GCTTGTCAATGGGCTT CTT; $Na_V1.7$: Fw: 5′ TGGCGTCGTGTCGCTTGT, Rv: 5′ TGGCCCTTT GCCTGAGAT; $Na_V1.8$: Fw: 5′ TCCTCTCACTGTTCCGCCTCAT, Rv: 5′ TTGCCTGGCTCTGCTCTTCATAC; $Na_V1.9$: Fw: 5′ ATACGGTGCCCT GATCCTCT, Rv: GGAAGTGAAGGGGCGGAAAT, Rpl29: Fw: 5′ ACAG AAATGGCATCAAGAAACCC, Rv: 5′ TCTTGTTGTGCTTCTTGGCAAA. The complementary DNA (cDNA) was added to the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific) with the forward and reverse primers described and nuclease-free water. To verify the results, we used no template negative controls and reverse transcriptase minus (RT-) negative controls. Amplification and quantification of the cDNA was carried out with the QuantStudio3 Real-Time PCR Instrument (Applied Biosystems) using the following thermal cycling conditions: 50°C for 2 min, 90°C for 10 min; 40 cycles of 95°C during 15 s and 60°C for 1 min; and final steps of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The expression level of the mRNA was normalized to the housekeeping Rpl29 mRNA levels and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6 | Immunoblotting

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). At DIV8, cell cultures were homogenized, dissolved in RIPA lysis buffer (50-mM HEPES, 140 mM NaCl, 10% glycerol, 1% v/v Triton X-100, 1 mM EDTA, 2 mM EGTA, 0.5% deoxycholate, pH 7.4) with EDTA-free protease inhibitor cocktail (cOmplete™, Sigma-Aldrich) and centrifuged at 18400 x *g* at 4ºC for 15 min. The supernatant was collected, and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Cellular protein extracts were mixed (1:1) with 2× sample buffer (0.125 mM Tris–HCl pH 6.8, 40 $mg{\cdot}ml^{-1}$ SDS, 2 $mg{\cdot}ml^{-1}$ bromophenol blue, 20% glycerol and 0.1-M dithiothreitol) boiled for 10 min. Thereafter, proteins were separated by electrophoresis on a 7.5% SDS-PAGE gel and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad) for 90 min at 100 V using an electrophoretic transfer system (Bio-Rad). Membranes were blocked with non-fat milk at 5% in TBST (200-mM glycine, 25 mM Tris base, 20% methanol and 0.05% Tween, pH 8.3) at room temperature for 1 h for all primary antibodies, except for TRPM8 that required 3-h blocking with non-fat milk and 0.5,h blocking with 5% BSA in TBST, according to manufacturer's instructions. Next, membranes were incubated at 4°C overnight with the following primary antibodies diluted in 1% BSA in TBST: rabbit anti-Actin, 1:1000 (Sigma-Aldrich, Cat# A2066, RRID:AB_476693); rabbit anti-TRPV1, 1:1000 (Alomone Labs, Cat# ACC-029); rabbit anti-TRPM8, 1:500 (Alomone Labs, Cat# ACC-049); rabbit anti-$Na_V1.7$, 1:2500 (Alomone Labs, Cat# ASC-008); and rabbit anti-$Na_V1.8$, 1:1000 (Alomone Labs, Cat# ASC-016), respectively. After washing the membranes with TBST, they were incubated with the secondary antibody anti-rabbit IgG, 1:20000 (Sigma-Aldrich, Cat# A0545, RRID:AB_257896). To minimize the number of animals used, blots were cut in three different sections to test the same blot with multiple antibodies. Signals were detected with the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific), visualized in a ChemiDoc™ MP Imaging System (Bio Rad ChemiDoc MP Imaging System, RRID:SCR_019037) and quantified using Image Lab software (Image Lab Software, BioRad, RRID:SCR_014210). Protein signals were normalized to actin levels within the same blot.

2.7 | Data and statistical analysis

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018), with the exceptions that data collection and analysis were not blinded because they were performed by the same researcher and, because effects and standard deviation could not be previously predicted, data were evaluated through experiments to minimize the number of animals used. Data were statistically analysed using the GraphPad Prism® 9.0.0 software (GraphPad Prism, RRID:SCR_002798). For quantitative variables, first we assessed whether data followed a gaussian distribution using D'Agostino-Pearson (omnibus k2) normality test. Data with a normal distribution are expressed as mean ± SD and analysed with unpaired *t* tests as indicated. As the heterogeneity of DRG neurons is high, and our sample size was not sufficient to determine multiple associations, we restricted our statistical analysis to the defined groups (treatment and sex type) that were analysed using the two-way ANOVA test for parametric data, with the Tukey post hoc test when *F* achieved statistical significance or Kruskal–Wallis with Dunn's multiple comparisons test for non-parametric data.

Non-normal distribution data are expressed as median, (with Q25–Q75) and analysed with Mann–Whitney test. For qualitative variables, data are expressed as percentage and analysed with Fisher's exact test. Significant differences were set to $P < 0.05$.

For patch clamp experiments, *n* represents the number of cells measured and correspond to the number of independent experiments, as a single neuron was monitored in each vehicle and paclitaxel-treated crystal; *N* denotes the number of rats used. For immunofluorescence measurements, *n* represents the number of neurons, and *N* the number of independent experiments; and for MEA, *n* denotes the number of active electrodes. Statistical analysis was based on the number of independent experiments. Details of the statistics are reported in the figure legends or Supporting Information.

2.8 | Materials

Paclitaxel (Taxol®, Tocris Bioscience, Bristol, UK) was dissolved in DMSO to create a stock of 25 mM; these stock solutions were stored at −20°C and used within 1 month. Before cell treatment, the stock solution was diluted in DMEM 10% FBS 1% P/S and passed through a 0.2-μm filter. The required quantity was added to the cell medium to yield a final concentration of 1 μM. Controls were prepared following the same procedure using only DMSO (0.04%).

For electrophysiological experiments, capsaicin, AITC, menthol, AMTB and PF04885614 (all from Sigma, St. Louis, MO, USA) were dissolved in DMSO to have stock concentrations of 10 mM, 1 M, 1 M, 100 mM and 100 μM, respectively. **ProTx II** (Tocris Bioscience) stock solution was prepared in water to a concentration of 50 μM. The day of the experiment, these solutions were diluted in the external solution to reach the final concentration indicated for each experiment. Final concentration of DMSO was 0.01% of the total volume for capsaicin, AITC, menthol and AMTB, and 0.075% for PF04885614.

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | Paclitaxel reversibly augmented the electrical excitability of small diameter DRG sensory neurons

The experimental paradigm consisted of seeding the sensory neurons (0 DIV) and, after their full maturation (5 DIV), treating them with 1 μM paclitaxel for 24 h (Figure 1a). Thereafter, the drug was removed, and the neural excitability was evaluated at 0 (6 DIV), 48 (8 DIV) and 96 h (10 DIV) after paclitaxel removal (post-treatment) (Figure 1a). As control, DRG cultures were exposed to paclitaxel vehicle (0.04% DMSO). We used 1 μM paclitaxel as a clinically relevant concentration (C_{max}) reached in a 24h infusion (Ohtsu et al., 1995). Incubation of neural cultures with paclitaxel for 24 h did not affect cell viability as neurons display normal RMPs, although it produced an axonal retraction (Figure 1b).

Paclitaxel increased the excitability of small diameter sensory neurons in a time-dependent manner, as shown by the larger number

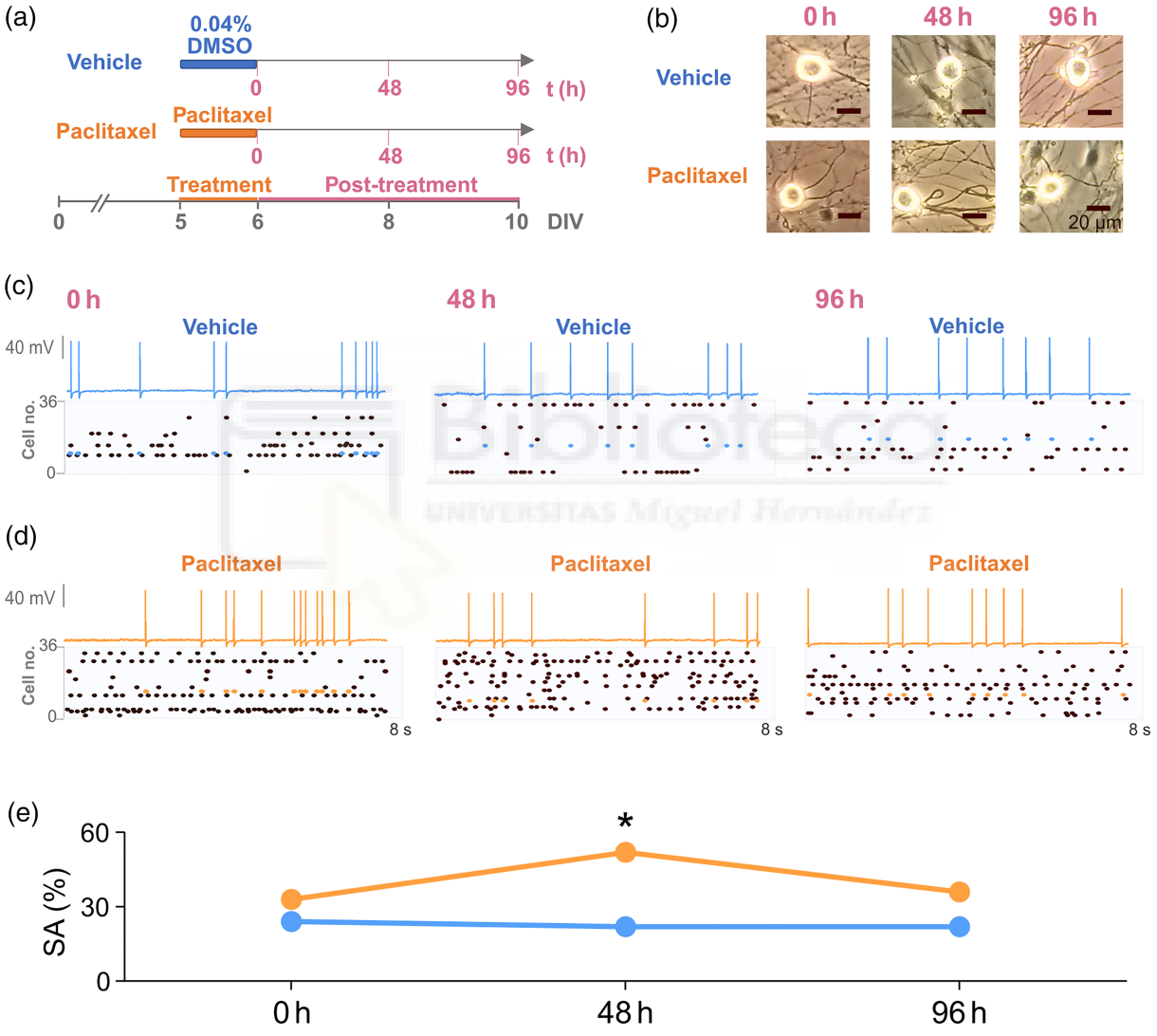

FIGURE 1 Small DRG neurons displayed increased excitability 48 h after paclitaxel treatment. (a) Diagram of the experimental design used for investigating the effect of paclitaxel on DRG neurons activity. Cells were incubated with the vehicle (0.04% DMSO) or with 1 μM paclitaxel during 24 h between days 5 and 6 of culture (5–6 DIV). Electrical recordings were performed at 0 h (DIV6), 48 h (DIV8) and 96 h (DIV10) post-treatment. (b) Representative images of the DRG neurons through the culture after paclitaxel exposure (lower panels) in comparison with vehicle condition (upper panels). (c and d) Representative recordings (top) and raster plot (bottom) of DRG neurons spontaneous firing of APs at 0, 48 and 96 h after vehicle (c) or paclitaxel (d) exposure. Each dot represents an individual spike fired during an 8 s protocol at 0 pA of current. The cell corresponding to the AP recording shown is blue coloured for vehicle and orange for paclitaxel-treated neurons in the raster plots. (e) Percentage of small DRG neurons exhibiting spontaneous activity (SA) at 0, 48 and 96 h after vehicle or paclitaxel treatment. Data are given as percentage with $N = 15$, $n = 34$ for vehicle and $n = 54$ for paclitaxel 24 h after exposure; $N = 20$, $n = 46$ for vehicle and $n = 54$ for paclitaxel, 48 h after exposure; $N = 19$, $n = 50$, 96 h after exposure. Results are shown as individual values with means ± SEM. *$P < 0.05$, significantly different from vehicle; Fisher's exact test

of sensory neurons exhibiting SA (Figure 1c,d, Table S1), that increased 2.4-fold at 48 h post-treatment and declined to virtually normal levels at 96 h (Figure 1e). We also observed a 1.8-fold increase in the percentage of small diameter sensory neurons exhibiting tonic firing, along with a 2-fold decrease in their current rheobase (Table S1). Thus, paclitaxel reversibly increased electrogenicity of sensory neurons, peaking at 48 h post-treatment and virtually dissipating at 96 h.

3.2 | Paclitaxel increased electrical firing in IB4(−) and IB4(+) sensory neurons

We next investigated the effect of paclitaxel on the electrical excitability of IB4(−) and IB4(+) neurons. In both neural populations, we observed a similar reversible effect of paclitaxel on their electrical activity, peaking at 48 h post-treatment (Tables S2 and S3). Paclitaxel increased the percentage of IB4(−) neurons displaying SA (Figure 2a) without changing the SA mean firing frequency (Figure 2c, Table S2). The chemotherapeutic drug significantly depolarized the RMP of IB4(−) nociceptors (Figure 2d). Similarly, IB4(+) neurons revealed a strong increase in the percentage of neurons exhibiting SA after paclitaxel exposure (Figure 2b,c, Table S3). We observed a tendency to RMP depolarization, but it did not reach statistical significance (Figure 2d). Cell capacitance and input resistance were not altered by paclitaxel in either neural population (Figure 2e,f).

We also evaluated paclitaxel effect on electrically evoked AP firing in both neural subtypes (Figure 3). As shown in Figure 3a, 55% of IB4(−) neurons recorded 48 h post-treatment exhibited tonic AP firing in response to a 120 pA current pulse (Table S2). Notably, paclitaxel augmented the firing frequency 2-fold (Figure 3a,b; Table S2) and significantly reduced the current rheobase (Figure 3b,e,f; Table S2). Notice that this increment in firing frequency was observed in the full range of currents from 10 to 300 pA (Figure 3b, Table S2), which resulted in an increment in the percentage of IB4(−) neurons exhibiting tonic firing (Figure 3g; Table S2). IB4(+) neurons primarily exhibited phasic AP firing when stimulated with a 120 pA current pulse. Paclitaxel increased 3-fold AP triggering (Figure 3c,d, Table S3), concomitant to a 3-fold decrease in the current rheobase, resulting in a 2-fold increment in the percentage of IB4(+) neurons exhibiting repetitive AP discharging after drug treatment (Figure 3g). These changes in the current rheobase and the percentage of IB4(+) neurons firing tonically disappeared 96 h after paclitaxel exposure (Table S3). Therefore, paclitaxel treatment of DRG cultures increased the electrogenic activity of both IB4(−) and IB4(+) neural subtypes. The effect on IB4(+) neurons is notable as they change from a silent and phasic to a tonic firing phenotype.

3.3 | Paclitaxel altered the electrical properties of IB4(−) and IB4(+) action potentials

The decrease in the current rheobase and the increase in repetitive firing in both IB4(−) and IB4(+) neurons produced by paclitaxel suggest an alteration in the AP waveform (Figure 4a). In control conditions, we observed that 58% of IB4(−) neurons exhibited a typical AP waveform with an inflection in the AP repolarization phase. Paclitaxel reduced this percentage to 27% of neurons. Paclitaxel also reduced the amplitude and accelerated the AHP phase. The t_r and the t_{AHP} were 32% and 22%

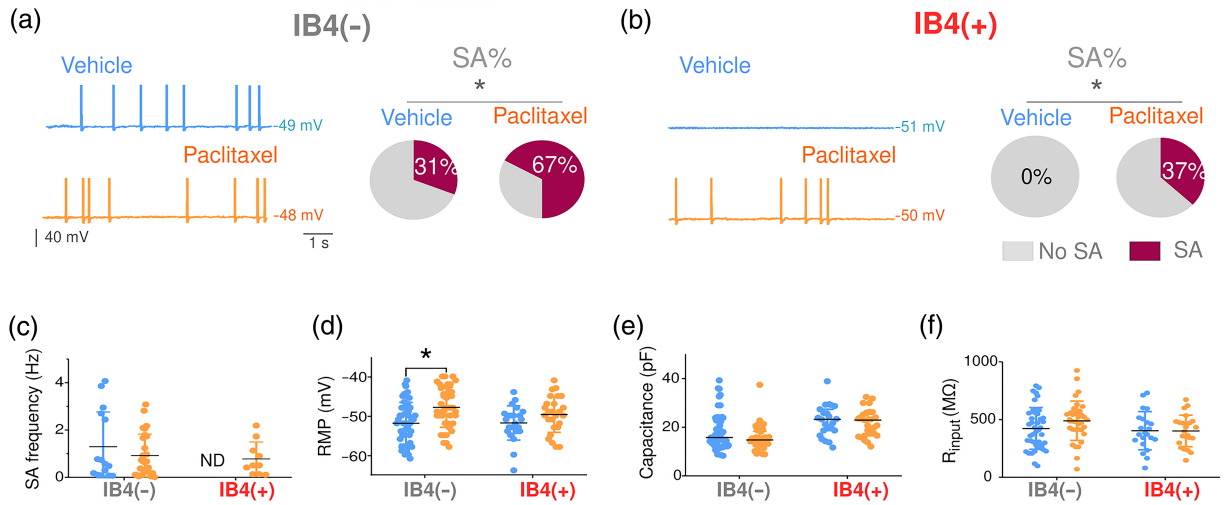

FIGURE 2 Paclitaxel increased the number of IB4(−) and IB4(+) neurons displaying spontaneous activity. (a and b) Representative AP recordings of IB4(−) (a) and IB4(+) (b) neurons 48 h after vehicle or paclitaxel treatment fired spontaneously (0 pA). The percentage of neurons firing SA for each group is indicated on the right as *pie charts*. The SA (%) is indicated inside the chart. (c) Frequency of spontaneous AP firing in 30 s recordings. Spontaneous activity was not detected in IB4(+) neurons treated with vehicle (ND). (d) Resting membrane potential (RMP) for IB4(−) and IB4(+) neurons at 48 h after vehicle or paclitaxel treatment. (e) Capacitance of the cells recorded in vehicle and paclitaxel groups for IB4(−) and IB4(+) was not statistically different. (f) Input resistance (R_{input}) remains unaltered for IB4(−) and IB4(+) neurons after paclitaxel exposure. Results are shown as individual values with means ± SD. $N = 20$, for IB4(−) with $n = 49$ for vehicle and $n = 42$ for paclitaxel; $N = 13$, for IB4(+) with $n = 25$. $^*P < 0.05$, significantly different from vehicle; Fisher's exact test (a and b), unpaired *t* test (c, d and f) or Mann–Whitney test (e)

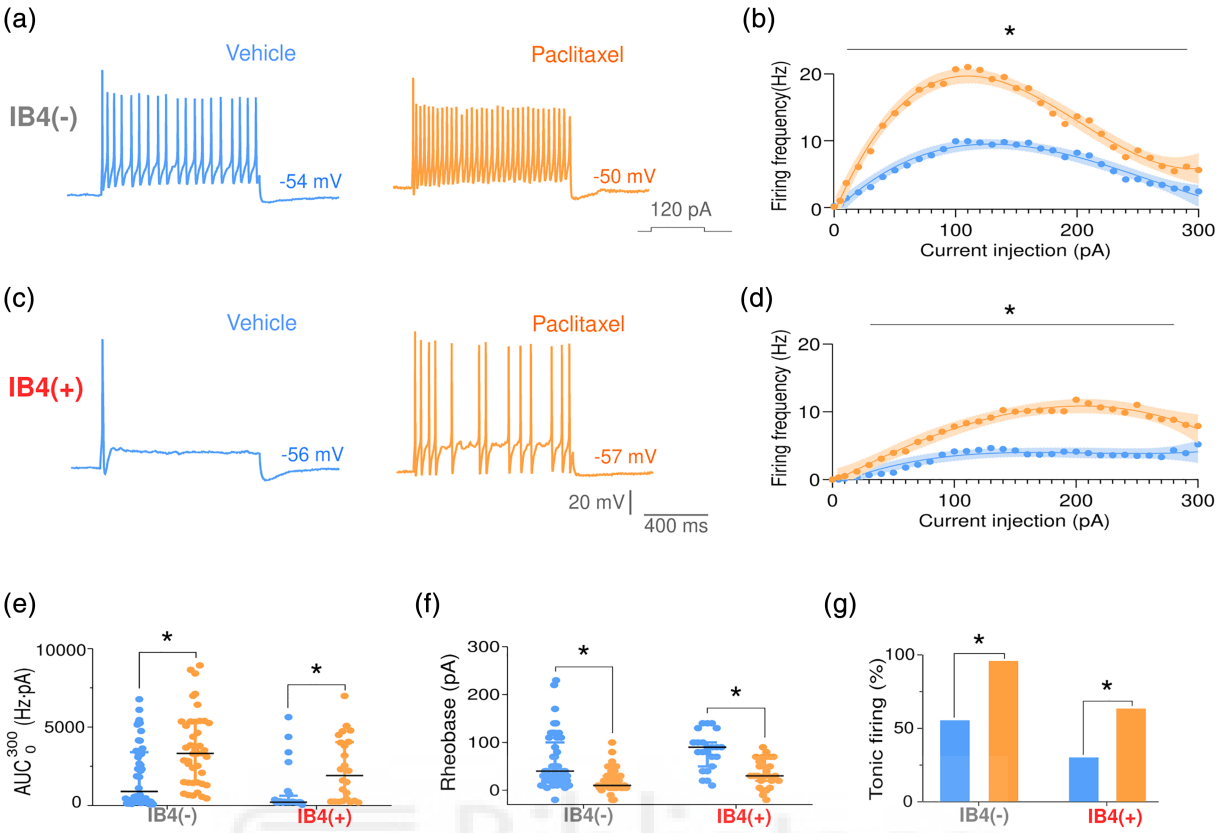

FIGURE 3 Paclitaxel potentiated electrically-evoked AP firing of IB4(−) and IB4(+) small-DRG neurons. (a and c) Representative recordings of AP firing evoked by a 1 s of current injection pulse of 120 pA for IB4(−) (a) and IB4(+) (c) neurons exposed to vehicle or paclitaxel recorded 48 h post-treatment. (b and d) Firing frequency (no. of APs evoked during 1 s depolarizing pulse) at each current injected from 0 to 300 pA in 10-pA intervals for IB4(−) (b) and IB4(+) (d) neurons exposed to vehicle or paclitaxel. (e) Area under the curve (AUC_0^{300}) obtained plotting the firing frequency as a function of the injected current in the range of 0 to 300 pA. (f) Rheobase values for IB4(−) and IB4(+) small DRG neurons exposed to vehicle or paclitaxel. (g) Percentage of neurons exposed to vehicle and paclitaxel exhibiting tonic firing. Data are expressed as individual values with medians, with interquartile ranges (IQR). Each dot represents the values measured for each cell recorded. Data were collected 48 h post-treatment. $N = 20$, for IB4(−) with $n = 49$ for vehicle and $n = 42$ for paclitaxel; $N = 13$, for IB4(+) with $n = 25$. *$P < 0.05$, significantly different from vehicle; Mann–Whitney test (b, d, e and f) or Fisher's exact test (g)

faster in drug treated IB4(−) neurons (Figure 4b). In addition, the AHP amplitude in these neurons was significantly reduced by 3 mV (Figure 4c). Other AP properties such as the t_{peak}, the V_{th}, overshoot, amplitude and the maximum upstroke slope were not altered by paclitaxel (Figure 4b–e). A similar analysis in IB4(+) neurons revealed that paclitaxel significantly depolarized the AHP amplitude by 3 mV (Figure 4h). All the other parameters did not evidence a statistically significant difference (Figure 4g–j). Thus, the higher electrical excitability observed in paclitaxel-treated IB4(−) and IB4(+) neurons appears primarily to arise from a smaller AHP and, in IB4(−) neurons, a faster repolarization and AHP recovery were also contributors.

3.4 | Paclitaxel increased Na_v channel currents in IB4(−) and IB4(+) neurons

IB4(−) sensory neurons displayed fast-inactivating inward currents upon 40 ms depolarizing pulses (Figure 5a), with a typical J-V curve for Na^+ ionic currents. The G-V relationship (Figure 5b) showed that paclitaxel hyperpolarized the $V_{1/2}$ and slightly altered the total Na^+ inward current, without altering the recovery from inactivation (Figure 5c). In contrast, paclitaxel did not affect the J-V nor the G-V curves of IB4(+) neurons (Figure 5d,e) but promoted a faster recovery of their Na^+ currents from inactivation (Figure 5f).

Because $Na_v1.7$ and $Na_v1.8$ channels have been shown to be altered by paclitaxel in animal models (Li et al., 2018; Zhang et al., 2018), we evaluated if the chemotherapeutic drug also affected them in our pre-clinical model. We used ProTx II and PF04885614 as specific blockers of $Na_v1.7$ and $Na_v1.7$ channels to study the contribution of these channels to Na^+ currents in IB4(−) and IB4(+) neurons (Figure 6). $Na_v1.7$ and $Na_v1.8$ ionic currents were obtained by subtracting the Na^+ ionic currents recorded in the presence of the blockers from that in their absence (Figure S1). In IB4(−) neurons, paclitaxel did not significantly alter $Na_v1.7$ currents (Figure 6a). A similar result was observed in IB4(+) neurons (Figure 6b). For $Na_v1.8$ channels, we observed an apparent current increase in IB4(+) nociceptors

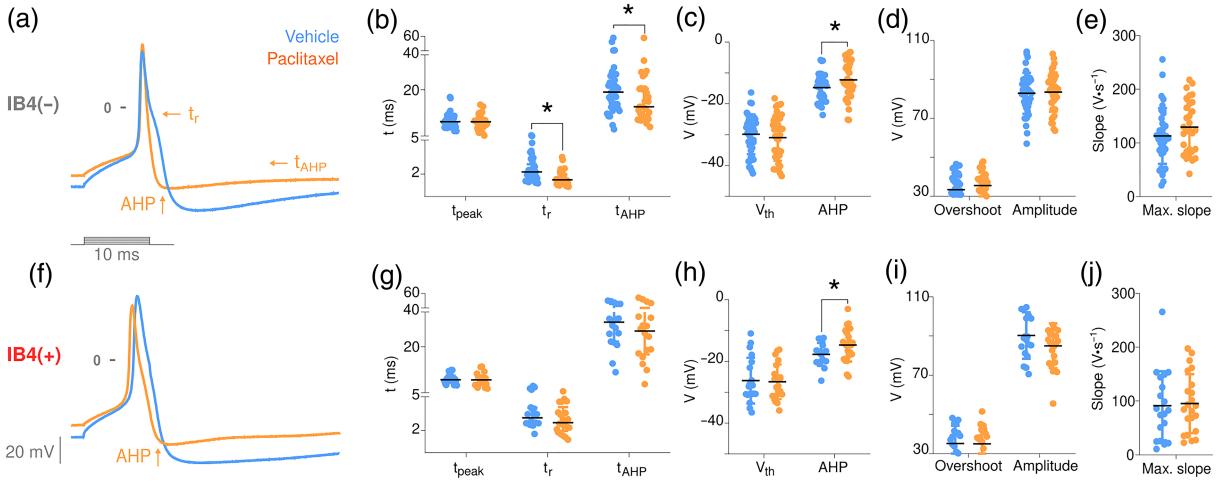

FIGURE 4 Paclitaxel altered the action potential parameters of IB4(−) and IB4(+) neurons. (a and f) Representative APs of IB4(−) (a) and IB4(+) (f) neurons exposed to vehicle or paclitaxel. Arrows mark action potential parameters in which statistical differences were found, as the repolarization time (t_r), the AHP amplitude (AHP) and the duration of the half AHP (t_{AHP}). APs were triggered using 10 ms current pulses from 0 to 300 pA and analysed at the current threshold. (b and g) Time to peak (t_{peak}), t_r and t_{AHP} parameters for IB4(−) (b) and IB4(+) neurons (g). t_r and t_{AHP} were significantly shorter in paclitaxel-treated IB4(−) neurons, whereas in IB4(+) neurons, there were no significant changes in t_r, t_{peak} and and t_{AHP}, following paclitaxel. (c and h) Action potential threshold (V_{th}) and AHP recorded for IB4(−) (c) and IB4(+) (h) neurons. Although the V_{th} was not affected by paclitaxel treatment in either set of neurons, the AHP amplitude was significantly increased by paclitaxel in both IB4(−) and IB4(+) neurons. (d and i) Overshoot and amplitude for IB4(−) (d) and IB4(+) (i). These parameters were not affected by paclitaxel treatment in either set of neurons. (e and j) Maximum upstroke slope (max. slope) for IB4(−) (e) and IB4(+) (j) neurons. This parameter was not affected by paclitaxel treatment in either set of neurons. Data were collected 48 h post-treatment. Values are expressed as mean ± SD. $N = 20$, $n = 37$ for vehicle, $n = 34$ for paclitaxel treated IB4(−) cells; $N = 10$, $n = 19$ for vehicle, $n = 22$ for paclitaxel IB4(+) cells. *$P < 0.05$, significantly different as indicated; unpaired t test or Mann–Whitney test (for t_{AHP} for IB4(−) and t_r for IB4(−) and IB4(+) cells)

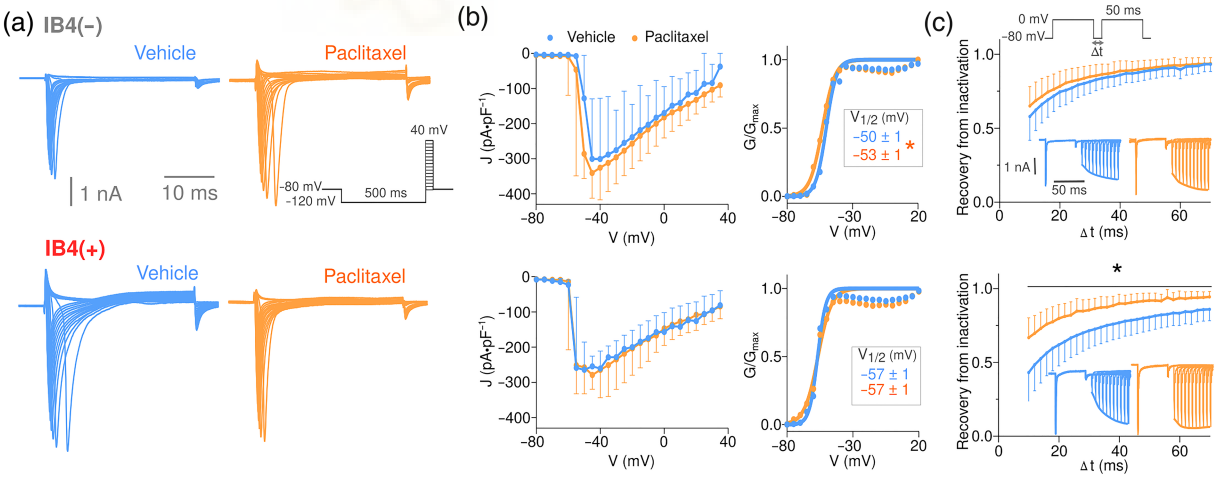

FIGURE 5 Na_V channel activity after exposure of IB4(−) and IB4(+) neurons to vehicle or paclitaxel. (a) Representative recordings of a family of ionic currents evoked using the 30 ms depolarizing protocol from −80 mV to 35 mV in 5 mV steps for IB4(−) and IB4(+) neurons 48 h after vehicle or paclitaxel treatment for IB4(−) (up) and IB4(+) (down) neurons. (b) J-V relationships of Na^+ inward currents present in the recordings for IB4(−) (up) and IB4(+) (down). J (pA·pF^{-1}) median values (with interquartile range) are shown. Right G-V curves obtained from the J-V curves. Reversal potential was interpolated from each J-V curve. Curves were fitted to the Boltzmann equation: $G/G_{max} = \left(1/\left(1+e^{-\frac{zF\left(V_{1/2}-V\right)}{RT}}\right)\right)$.

(c) Recovery from inactivation of the Na_V currents against time for IB4(−) (up) and IB4(+) (down). Representative registers of the Na_V recovery from inactivation are indicated in each graph. Data are expressed as medians (with interquartile range). Data were collected 48 h post-treatment. IB4(−): $N = 5$, $n = 18$ for vehicle and $n = 19$ for paclitaxel; IB4(+): $N = 5$, $n = 16$ for vehicle and $n = 18$ for paclitaxel. *$P < 0.05$, significantly different from vehicle; Mann–Whitney test for J, unpaired t test for recovery from inactivation

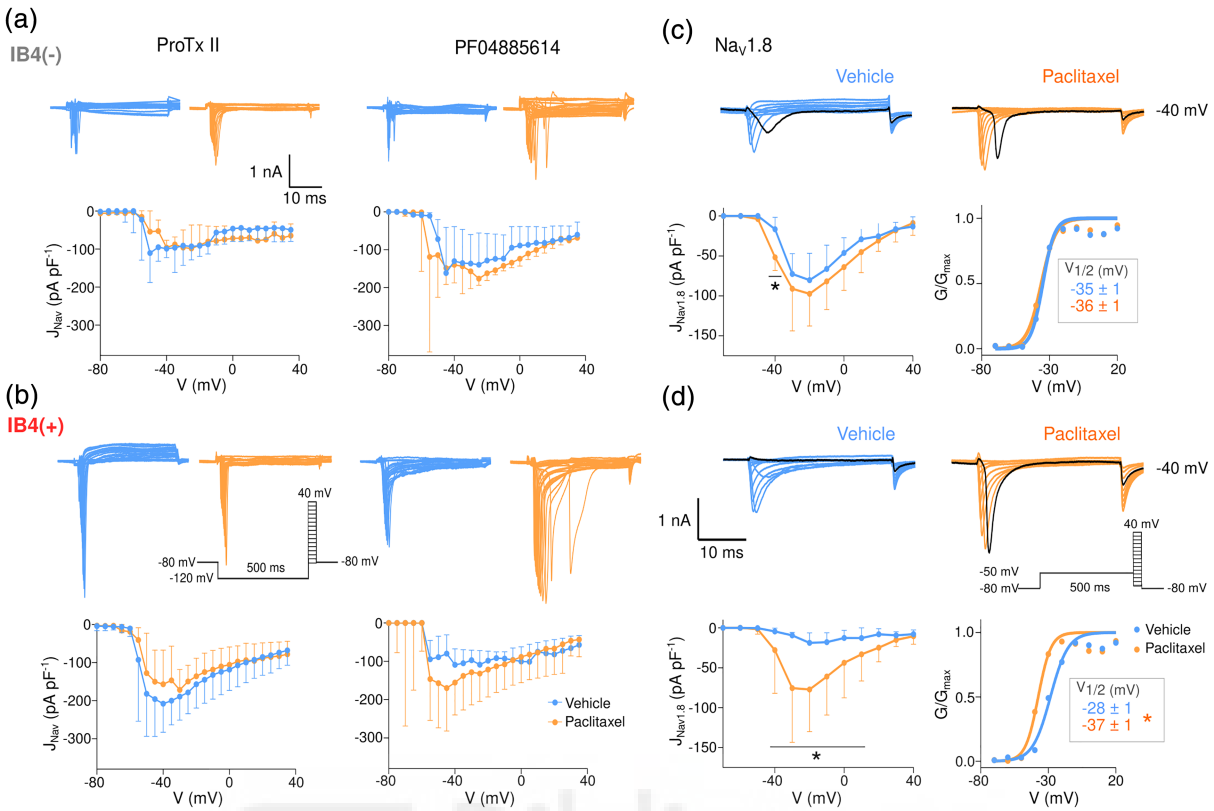

FIGURE 6 Paclitaxel augmented $Na_V1.8$ currents in IB4(−) and IB4(+) sensory neurons. (a and b) Pharmacological dissection of vehicle and paclitaxel Na_V currents using the specific blockers: ProTX II at 10 nM for $Na_V1.7$ channels and PF04885614 at 75 nM for $Na_V1.8$ channels. The current after blockade was subtracted from the total current measured. J-V relationships of Na^+ inward currents for each channel indicated below their respective representative registers for IB4(−) (a) and IB4(+) (b). J ($pA{\cdot}pF^{-1}$) median values (with interquartile range) are shown. $N = 5$, $n = 6$ for each blocker. (c and d) Representative registers of the isolated $Na_V1.8$ currents activated using a voltage protocol from −70 to 40 mV in 10 mV intervals. Black lines indicate the ionic current detected at −40 mV for IB4(−) (c) and IB4 (+) (d). JV and GV curves for $Na_V1.8$ current are exhibited below the recordings. $V_{1/2}$ values are indicated inside squares. Data are expressed as medians (with interquartile range). Data were collected 48 h post-treatment. $N = 7$. IB4(−): $n = 17$ for vehicle, $n = 19$ for paclitaxel; IB4(+): $n = 17$ for vehicle, $n = 21$ for paclitaxel. $^*P < 0.05$, significantly different from vehicle; Mann–Whitney test for J and the unpaired t test for $V_{1/2}$

(Figure 6b) that was less evident in IB4(−) sensory neurons (Figure 6a). To further interrogate the potential alteration of $Na_v1.8$ currents, we applied a stimulation protocol consisting in a 500-ms depolarizing pre-pulse to −50 mV to inactivate TTX-sensitive and TTX-resistant Na_v channels, followed by a family of 30-ms depolarizing 10 mV step potentials from −70 mV to 40 mV to monitor the activity of $Na_v1.8$ channels (Figure 6c,d) (Soriano et al., 2019). In IB4(−) neurons, paclitaxel produced a modest increase in $Na_v1.8$ ionic currents (Figure 6c). In contrast, paclitaxel produced a significant increase in $Na_v1.8$ ionic currents in IB4(+) neurons (Figure 6d) that was accompanied by a 9 mV hyperpolarizing shift of the $V_{1/2}$ (Figure 6d). Furthermore, treatment of IB4(+) neurons with PF04885614 inhibited the fast recovery from inactivation promoted by paclitaxel (Figure S2). To substantiate these findings, we evaluated the expression of these channels. mRNA levels of $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ in nociceptor cultures were not significantly affected by paclitaxel (Table S5). Similarly, we did not detect a significant increase in the protein expression in whole cell extracts evaluated by immunoblotting (Figure S3). However, immunocytochemical measurements revealed a significant increase in $Na_v1.8$ immunoreactivity in sensory neurons treated with paclitaxel consistent with an increment in protein expression in a subset of neurons (Figure S4). Taken together, these results imply that in our in vitro nociceptor model paclitaxel primarily appears to increase the expression and function of $Na_v1.8$ channels.

3.5 | Paclitaxel affected A-type K^+ currents in IB4(−) and IB4(+) DRG neurons

We also studied the effect of paclitaxel on K^+ currents in IB4(−) and IB4(+) neurons. In IB4(−) neurons, depolarizing 10-mV voltage pulses from −80 to 40 mV evoked a family of outwardly rectifying, non-inactivating K^+ currents (K_{DR}). Paclitaxel produced outwardly rectifying K^+ currents that displayed two discernible components, a fast-inactivating A-type current (K_A) followed by a K_{DR} current, clearly shown at depolarizing potentials ≥−30 mV (Figure 7a). Their distinct kinetics allowed us to analyse both K^+ currents (McFarlane & Cooper, 1991). The J-V

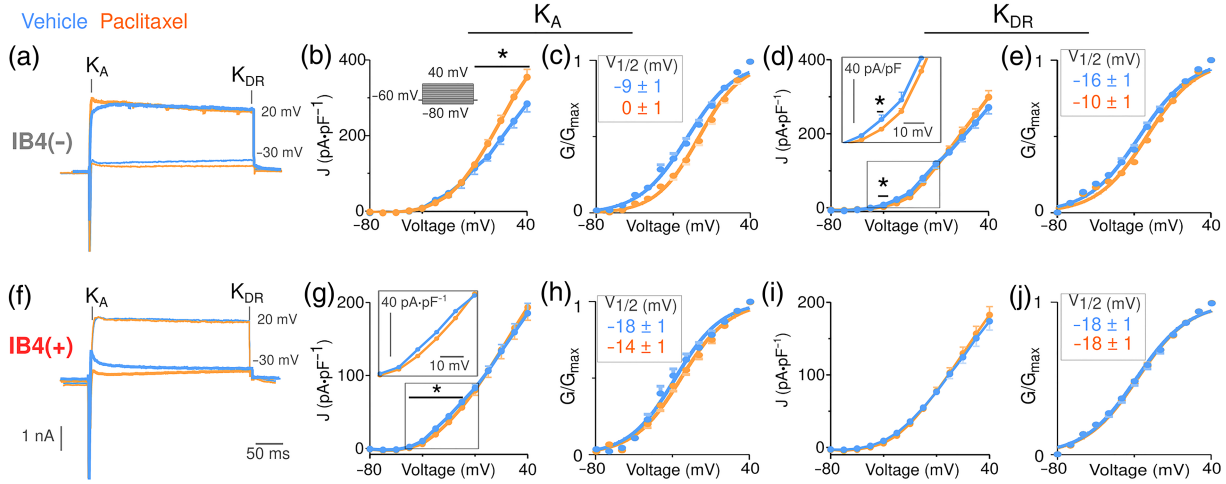

FIGURE 7 Paclitaxel affected the fast-inactivating (K_A) component of K^+ outward currents in IB4(−) and IB4(+) neurons. (a and f) Representative recordings of ionic currents in response to a depolarizing pulse to 20 mV and −30 mV for IB4(−) (a) and IB4 (+) (f) neurons. Note that a fast-inactivating (K_A) and non-inactivating (K_{DR}) components can be distinguished. (b, d, g and i) J-V relationships of K_A and K_{DR} components in IB4(−) (b and d) and IB4(+) (g and i). Current densities (pA·pF^{-1}) are given as median values with interquartile range. (c, e, h and j) G-V curves obtained from IV curves for K_A and K_{DR} for IB4(−) (c and e) and IB4(+) (h and j) using a $V_{K^+} = -92$ mV. Mean values with SEM are shown and curves were fitted to Boltzmann equation (see Figure 5 legend) with the following parameters: for IB4(−) K_A (c): Vehicle: $z = 1.4$ e_0, $df = 546$, $R^2 = 0.85$; paclitaxel: $z = 1.5$ e_0, $df = 440$, $R^2 = 0.9$; for K_{DR} (e): Vehicle: $z = 1.3$ e_0, $df = 559$, $R^2 = 0.9$; paclitaxel: $z = 1.3$ e_0, $df = 557$, $R^2 = 0.9$; for IB4(+) K_A (h): Vehicle: $z = 1.4$ e_0, $df = 258$, $R^2 = 0.9$; paclitaxel: $z = 1.4$ e_0, $df = 348$, $R^2 = 0.9$; for K_{DR} (j): Vehicle: $z = 1.3$ e_0, $df = 245$, $R^2 = 0.9$; paclitaxel: $z = 1.3$ e_0, $df = 349$, $R^2 = 1.0$ half-maximum activation voltages ($V_{1/2}$) for each G-V curve are indicated inside a square on each graph. Data were collected 48 h post-treatment. IB4(−): $N = 15$, $n = 28$ for vehicle and $n = 33$ for paclitaxel. IB4(+): $N = 13$, $n = 20$ for vehicle, $n = 27$ for paclitaxel. *$P < 0.05$, significantly different from vehicle; Mann–Whitney test

relationship for K_A currents showed that paclitaxel significantly increased the magnitude of this component at V ≥ 10 mV (Figure 7b). Paclitaxel effect on K_A currents could be larger as it may be partially obscured by the presence of the K_{DR} currents. The G-V curve was shifted to depolarizing potentials, resulting in a 9 mV right-shift of the $V_{1/2}$ (Figure 7c). In contrast, the magnitude of K_{DR} currents was not significantly affected by paclitaxel (Figure 7d), whereas the G-V curve appeared depolarized by 6 mV, most likely because of the change in the K_A current.

In IB4(+) neurons, depolarizing voltage pulses elicited both K_A and K_{DR} currents (Figure 7f). Paclitaxel reduced 20% the K_A current magnitude (Figure 7g,i) but did not alter the $V_{1/2}$ (Figure 7h,j). Therefore, the drug promoted the appearance of a high threshold K_A current in IB4(−) neurons, while in IB4(+) neurons, it reduced a low threshold K_A current.

3.6 | Paclitaxel sensitized IB4(−) and IB4(+) neurons from both female and male rats

Next, we evaluated whether there was any sign of sex dimorphism in the neural sensitization by paclitaxel (Figure 8). Paclitaxel augmented 2-fold the percentage of IB4(−) neurons of both sexes exhibiting SA (Table S4), as well as their tonic firing (Figure 8a,b). Paclitaxel intensified the firing activity of female IB4(−) neurons by 2.2-fold, while for male neurons there was a 1.5-fold increase. The peak of the bell-shaped curve in female neurons was shifted towards lower current values (Figure 8c), implying a stronger sensitizing effect in female IB4(−) neurons (Figure 8g, Table S4). Furthermore, paclitaxel significantly reduced the current rheobase in female IB4(−) neurons (Figure 8H, Table S4) and depolarized the RMP by 4 mV (Figure 8i, Table S4). In male IB4(−) neurons, the effect of paclitaxel on the current rheobase and RMP did not reach statistical significance.

In IB4(+) neurons, paclitaxel increased the percentage of neurons displaying SA in both sexes, by up to 40% (Table S4). Noteworthy, the drug increased ≥3.0-fold tonic firing in female IB4(+) neurons and 1.5-fold in male (Figure 8d,g). This augment of AP firing in female IB4(+) neurons was also accompanied by a 3-fold decrease of the current rheobase (Figure 8h, Table S4). The drug also depolarized the RMP of female IB4(+) neurons as compared to male neurons, although it did not reach statistical significance (Figure 8l, Table S4). Thus, small sensory neurons from female rats appear more sensitive to paclitaxel potentiation than those from males.

3.7 | Paclitaxel increased TRPV1 and TRPM8 channel functionality

TRPV1, TRPM8 and TRPA1 are thermoTRP channels that may be implicated in the thermal hypersensitivity experienced by CIPN patients (Nazıroğlu & Braidy, 2017). Multielectrode array measurements revealed that paclitaxel augmented by ≥3-fold the electrogenic responses to capsaicin or menthol, without altering TRPA1 channel activity (Figure S5), suggesting an increment in the expression of TRPV1 and TRPM8 channels. However, analysis of their mRNA

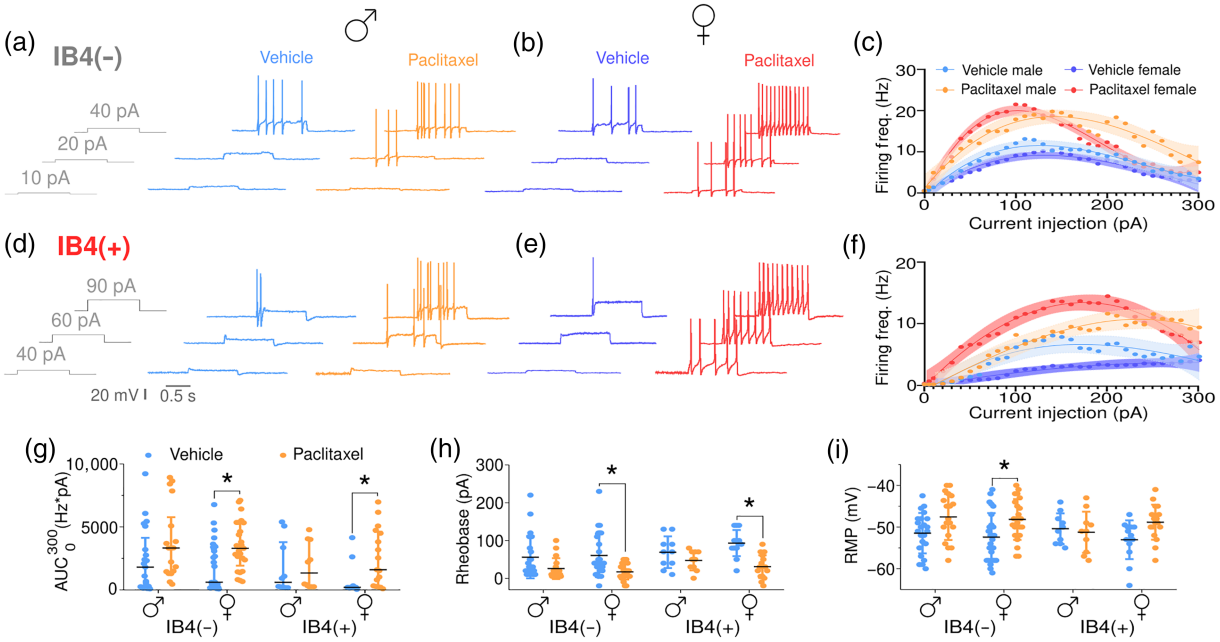

FIGURE 8 Neural excitability of IB4(−) and IB4(+) neurons from female rats appear more sensitive to paclitaxel. (a, b, d and e) Representative action potential firing of IB4(−) and IB4(+) neurons from female (b and e) and male (a and d) rats evoked by 1 s current pulse as indicated on the left side. (c and f) Firing frequency as a function of the injected current in the range of 0 to 300 pA in 10-pA pulses. Firing frequency was obtained counting the number fired APs during the 1 s current pulse. (g) Area under the curve (AUC_0^{300}) obtained by integrating the firing frequency curve for the 0 to 300 pA current interval in female and male neurons. (h) Rheobase values estimated as the minimum injected current need to fire an action potential in female and male neurons. (i) RMP measured using a continuous current protocol of 0 pA in female and male neurons. Data are presented as individual values and the means ± SD. Data were collected 48 h post-treatment. Male IB4(−): $N = 9$, vehicle: $n = 23$, paclitaxel: $n = 19$; female IB4(−): $N = 11$, vehicle: $n = 26$, paclitaxel: $n = 23$; male IB4(+): vehicle: $n = 10$, paclitaxel: $n = 10$; female IB4(+): vehicle: $n = 13$, paclitaxel: $n = 17$. IB4(−) and IB4(+) neurons were analysed separately. *$P < 0.05$, significantly different as indicated; Kruskal–Wallis test with Dunn's multiple comparisons test (g, h) or two-way ANOVA with Tukey's multiple comparisons test (i)

expression (Table S5) did not show a transcriptional regulation by paclitaxel of these channels. At a protein level, we observed a significant increase of TRPV1 expression (Figure S3). Similarly, immunocytochemical analysis of sensory neurons revealed a significant increase of TRPV1 immunoreactivity (Figure 9b,c). For TRPM8, we observed an increase in immunoreactivity (Figure 9h,i) that was not substantiated by immunoblots (Figure S3). TRPA1 immunoreactivity was not affected by paclitaxel (Figure 9e,f).

We next evaluated the effect of paclitaxel on the ionic currents induced by capsaicin, menthol and AITC in IB4(−) and IB4(+) neurons (Figure 10; Table S6). Figure 10A illustrates representative capsaicin inward currents elicited in IB4(−) and IB4(+) neurons exposed to vehicle and paclitaxel. Capsaicin-evoked ionic currents were 4-fold augmented in IB4(−) neurons and mildly promoted in IB4(+) neurons treated with the drug (Figure 10B). As expected, paclitaxel did not affect the AITC-evoked ionic currents in either neural population (Figure 10c,d). In contrast, paclitaxel increased 3-fold the menthol response in IB4(−) neurons (Figure 10e,f). No menthol currents were recorded from IB4(+) neurons. Thus, these results indicate that paclitaxel mainly increased TRPV1 and TRPM8 channel activity in IB4(−) neurons.

We also investigated if paclitaxel potentiation of TRPV1 and TRPM8 activity displayed sexual dimorphism. Paclitaxel similarly increased capsaicin-evoked ionic currents in male and female IB4(−) neurons by ≥3.5-fold (Figure 10g,h). In contrast to the effects obtained with capsaicin, paclitaxel augmented menthol-evoked ionic currents 4.5-fold in male IB4(−) neurons but showed no significant increase in female IB4(−) neurons (Figure 10i,j). Paclitaxel similarly affected the capsaicin currents of IB4(+) male and female neurons (Figure 10k,l). Accordingly, paclitaxel similarly potentiated capsaicin responses in male and female sensory neurons, but the responses to menthol were potentiated only in male IB4(−) neurons.

4 | DISCUSSION

We have examined the direct effect of paclitaxel on nociceptor excitability using a long-term, in vitro, pre-clinical model of nociception that allowed us to study the reversibility of paclitaxel-induced neural sensitization. A salient finding was that paclitaxel exposure (24 h) increased SA and tonic firing of IB4(−) and IB4(+) sensory neurons, peaking at 48 h and dissipating at 96 h post-treatment. Potentiation of the electrogenic activity mainly resulted from a reduction of the current rheobase in IB4(−) and IB4(+) neurons, and a modest RMP depolarization in IB4(−) neurons. IB4(+) neurons shifted from a phasic to an irregular tonic firing, while in IB4(−) neurons, the tonic firing

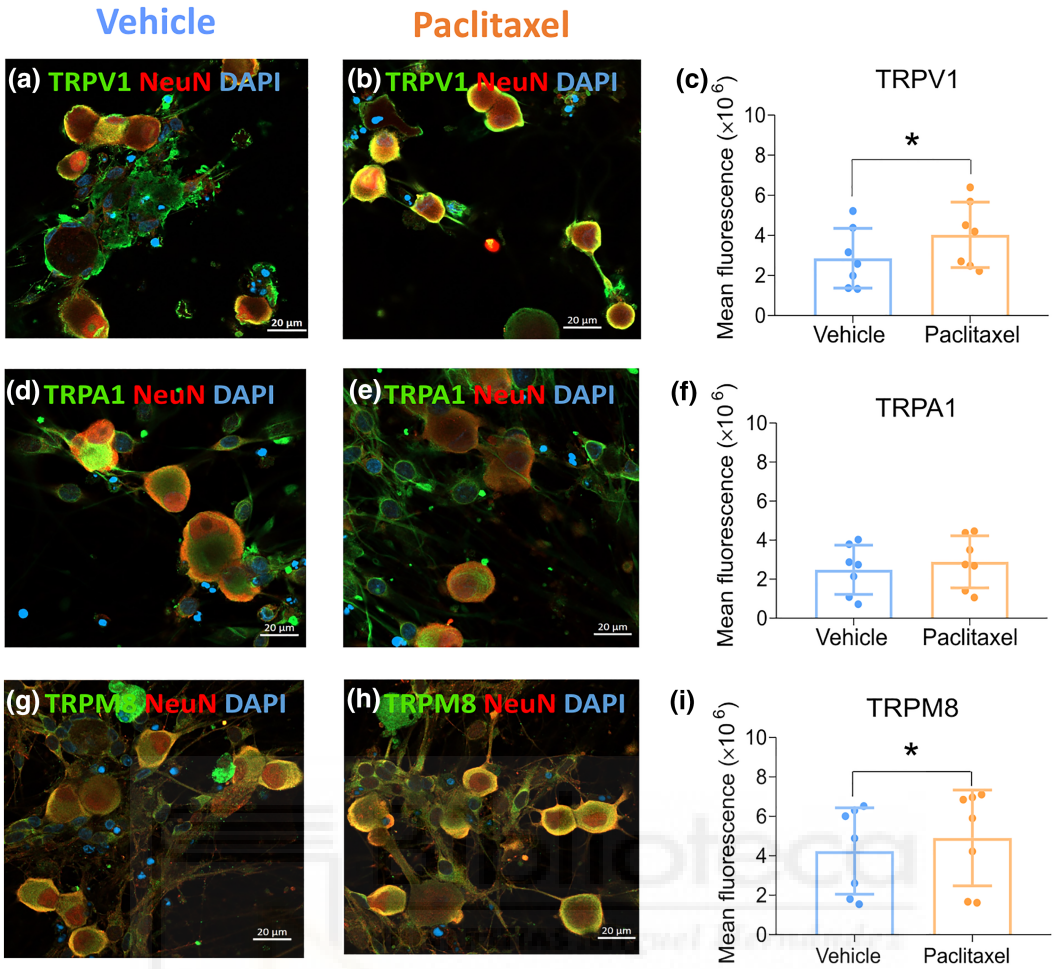

FIGURE 9 Paclitaxel increased immunoreactivity for TRPV1 and TRPM8 in sensory neurons. Immunocytochemical staining of cultured DRG neurons treated with vehicle or paclitaxel using anti-TRPV1, anti-TRPA1 or anti-TRPM8 antibodies along with the neuronal marker NeuN and DAPI staining. Green: TRPV1 (a, b), TRPA1 (d, e) or TRPM8 (g and h). Red: NeuN. DAPI: cyan. Scale bar denotes 20 µm. (c, f and i) Mean fluorescence intensity of TRPV1 (c), TRPA1 (f) or TRPM8 (i) immunoreactivity for vehicle and paclitaxel treated neurons. Data were collected 48 h post-treatment. Data are presented as individual values with means ± SD; $N = 7$. *$P < 0.05$, significantly different from vehicle; paired t test

was potentiated. Analysis of the AP waveform indicated that electrogenic changes in IB4(−) were due to faster AP repolarization and to a faster and shallower AHP, while the effects observed in IB4(+) neurons resulted from down-regulation of the AHP amplitude. These results confirm that paclitaxel-induced electrogenic sensitization of DRG neurons does not require the participation of immune cells, although an autocrine action of IL-6 and CCK2 could be a contributory factor (Miller et al., 2009).

Several studies have shown an alteration of neural Na^+ currents in CIPN due to paclitaxel (Nieto et al., 2008; Zhang & Dougherty, 2014) that mainly arises from an up-regulation of Nav1.7 channels in sensory neurons (Li et al., 2018). $Na_v1.7$ channels are TTX-sensitive channels displaying a hyperpolarized activation potential that contributes to set the AP threshold and the current rheobase in nociceptors (Dib-Hajj et al., 2005). Furthermore, the ratio of $Na_v1.7$: $Na_v1.8$ expression has been proposed to modulate the amplitude of subthreshold oscillations that may promote repetitive firing and control electrogenicity (Choi & Waxman, 2011). $Na_v1.8$ channels are TTX-resistant Nav channels mainly involved in setting the upstroke of the AP, defining the inflection of the repolarization phase occurring in 60% of IB4(−) neurons, tuning the fast recovery from inactivation and contributing to the generation of resurgent Na^+ currents (Renganathan et al., 2001; Xiao et al., 2019). Recent studies have shown that paclitaxel hyperexcitability could be blocked by the $Na_v1.8$-specific antagonist **A-803467** (Verma et al., 2020). Similarly, the work of Zhang et al. suggested that puerarin reduced paclitaxel induced neuropathic pain in rats by blocking the $Na_v1.8$ β1 subunit (Zhang et al., 2018). Our findings also point to $Na_v1.8$ channels in both nociceptor subtypes, as a central player of paclitaxel-induced neural excitability. The greater increase of $Na_v1.8$ channel activity in IB4(+) nociceptors treated with paclitaxel is consistent with the faster recovery of inactivation of Na^+ ionic currents observed in these nociceptors. In support of this proposal, treatment of IB4(+) nociceptors with PF04885614 eliminated the faster recovery from inactivation provoked by paclitaxel. Intriguingly, in our model we did not observe any increase of $Na_v1.7$ channel activity, as reported in nociceptors from other animal models of

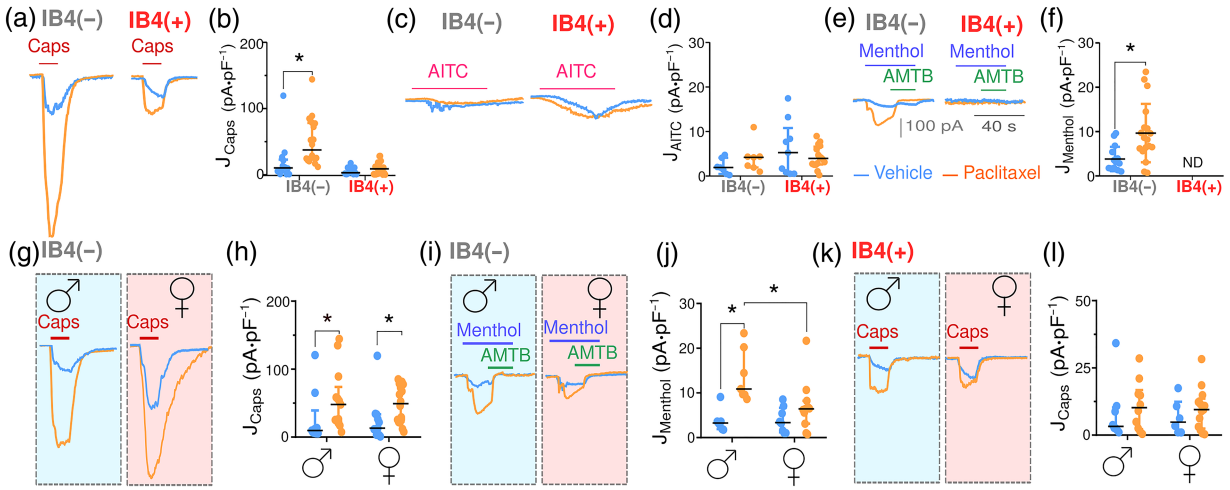

FIGURE 10 Paclitaxel increases TRPV1 and TRPM8 channel activity in IB4(−) neurons. (a, c and e) Representative inward current responses to a 1 μM capsaicin (a), 100 μM AITC (c) and 100 μM menthol (e) of IB4(−) and IB4(+) neurons exposed to vehicle or paclitaxel. Calibration bars are for all recordings. TRPM8 channel activity was further confirmed by using the antagonist 10 μM AMTB along with the menthol pulse. (b, d, and f) Peak current density observed after application of capsaicin (b), AITC (d) or menthol (f). AITC current was evoked in the same cells as capsaicin, after application of 4 capsaicin pulses. To quantify TRPM8 responses the remaining inward current during co-application of menthol and AMTB was subtracted from the peak response to menthol. Data are expressed as medians (with interquartile range) or as mean ± SD. $^*P < 0.05$, significantly different as indicated; Mann–Whitney test for capsaicin and AITC or unpaired *t* test for menthol. (g, i and k) Representative current responses to 1 μM capsaicin (g) and 100 μM menthol (i) for IB4(−) neurons and to capsaicin for IB4(+) (k) from male and female rats. (h) Quantitation of the current density in response to 1 μM capsaicin revealed a similar potentiating effect of paclitaxel evoked in male DRG neurons, and their female counterparts. (j) Menthol-activated ionic currents were activated more potently in male IB4(−) DRG neurons ($N = 4$, $n = 7$), than in female IB4(−) neurons ($N = 4$, $n = 8$ for vehicle, $n = 11$ for paclitaxel). $^*P < 0.05$, significantly different as indicated. (l) There was no significant effect of paclitaxel on the capsaicin-evoked currents in neurons from male rats ($N = 5$, $n = 8$ for vehicle and $n = 10$ for paclitaxel) or those from females ($N = 6$, $n = 6$ for vehicle and $n = 13$ for paclitaxel), although there was a trend towards an increase in both male and female cells. Recordings were made 48 h post-treatment. Data are presented as individual values with medians and interquartile ranges. $^*P < 0.05$, significantly different as indicated; Kruskal–Wallis with Dunn's multiple comparisons test

paclitaxel-induced CIPN (Li et al., 2018; Xia et al., 2016). A possible explanation is that modulation of $Na_v1.7$ channel activity in nociceptors by paclitaxel may require the participation of pro-algesic agents released from immune cells affected by the drug or/and that paclitaxel increased the axonal location of the channel (Akin et al., 2021). Thus, our findings suggest that $Na_v1.8$ channels are the major players defining the electrogenic properties following exposure to paclitaxel, including the threshold of action potential firing and the occurrence of repetitive firing (Choi et al., 2007; McDermott et al., 2019). Nonetheless, additional experiments are needed to define the contribution of $Na_v1.7$ and $Na_v1.9$ channels to setting the RMP and to regulate the neuronal excitability of different subpopulations of sensory neurons.

Electrogenicity is also defined by the AHP phase of the AP that is shaped by HCN (**HCN1** and/or **HCN2**), **K_{2P}** and **K_{ir}** channels. Down-regulation of K_{2P} and K_{ir} channels would reduce the AHP amplitude depolarizing the RMP and increase neural excitability. Transcriptional analysis of DRGs from paclitaxel-treated mice showed a down-regulation of the mRNA for **KCN1 ($K_v1.1$)** and **$K_{ir}3.4$** (Zhang & Dougherty, 2014), which is consistent with the smaller AHP amplitude we observed. Because of their Na^+ permeability, HCN channels modulate AHP dynamics and electrogenic adaptation (Gu et al., 2005). An up-regulation of HCN channels reduces AHP amplitude and accelerates restoration of RMP (Emery et al., 2012), thus promoting repetitive firing (Hogan & Poroli, 2008). Up-regulation of HCN2 channels has been shown in inflammatory and neuropathic pain conditions (Liu et al., 2018; Weng et al., 2012). Furthermore, a transcriptional increase of HCN1 mRNA expression was reported in an in vivo model of paclitaxel CIPN (Zhang & Dougherty, 2014), and HCN1 blockade attenuated oxaliplatin-induced CIPN (Resta et al., 2018). Accordingly, our finding that paclitaxel down-regulates AHP in IB4(−) and IB4(+) neurons suggests an up-regulation of HCN channels, most likely HCN1 (Zhang & Dougherty, 2014), although HCN2 cannot be fully ruled out. Collectively, our findings suggest that the effects of paclitaxel on IB4(−) and IB4(+) electrogenicity are due to regulation of Na_v and HCN channels, and probably K_{2P} and K_{ir} channels. Nonetheless, we cannot discard regulation of other channels that also contribute to the AP waveform and electrogenicity, including Ca^{2+}-activated K^+ channels (BK, IK, SK) that are expressed in sensory neurons (Tsantoulas & McMahon, 2014). For instance, in IB4(−) neurons the faster AP repolarization may result from the up-regulation of a high-threshold K_A channel such as $K_v4.3$ that is a major contributor to K_A currents in IB4(−) nociceptors (Zemel et al., 2018). In IB4(+) neurons, AP repolarization could be influenced by the down-regulation of a low threshold K_A channel, such as $K_v1.4$ that is highly expressed in these neurons (Vydyanathan et al., 2005). Additional experiments are

needed to identify the ion channels altered by paclitaxel and to analyse how the drug affects their function (Malacrida et al., 2019).

We found that paclitaxel also up-regulated TRPV1 and TRPM8 channels, implying their contribution to heat and cold hypersensitivity in paclitaxel-evoked CIPN. Aligned with our findings, up-regulation of TRPV1 channel function by paclitaxel was previously observed and related to the burning pain that patients experience (Hara et al., 2013; Li et al., 2015). Our data suggest that paclitaxel-induced cold allodynia mainly arises from an up-regulation of TRPM8 channels. This result is consistent with a previous report showing that AMTB, a TRPM8 channel antagonist, reduced cold-hyperalgesia exhibited by paclitaxel-treated mice (Sałat & Filipek, 2015). However, our finding contrasts with the suggestion that paclitaxel-induced cold allodynia may be mediated by TRPA1 channels (Nazıroğlu & Braidy, 2017). A study in diabetic rats showed that paclitaxel up-regulated TRPA1 channels in sensory neurons and proposed a major role in cold allodynia in CIPN (Barrière et al., 2012). Intriguingly, we did not observe a direct effect of paclitaxel in the expression or function of TRPA1 channels. This discrepancy may arise from the different CIPN models used, as up-regulation of TRPA1 channels in vivo may be not due to the direct action of the drugs, but rather by algesic molecules released from immune cells (Materazzi et al., 2012; Viana, 2016).

Another relevant result of our preclinical in vitro nociceptor model is the finding that sensory neurons from females exhibit an apparently greater sensitivity to paclitaxel than those from males, suggesting a sex dimorphism. In contrast to the results in neurons from, paclitaxel provoked, in female neurons, a significant change in firing frequency, current rheobase and RMP depolarization, indicating a stronger drug-induced sensitization. However, sex-related differences in the electrogenic parameters did not reach statistical significance, most likely due to the modest sample size for the large phenotypic heterogeneity of nociceptors. Unexpectedly, we observed that paclitaxel promoted a higher up-regulation of TRPM8 channel activity in male sensory neurons than in females, while the effect on TRPV1 channel function was similar in both sexes. Studies in animal models have also reported the existence of sex dimorphism in paclitaxel CIPN, although these studies implied an immune-directed mechanism through **TLR9**-mediated nociceptor sensitization (Luo et al., 2019). Our findings additionally indicate that differences in sensitivity between male and female sensory neurons to paclitaxel are possible contributors to sex dimorphism. Studies that explore the sex prevalence of paclitaxel in humans also hint to higher prevalence in women, although the number of studies is limited, and these data need to be treated with caution (Kim et al., 2018). Nonetheless, the potential sex dimorphism in paclitaxel-induced sensitization of DRG neurons deserve further investigation and strongly supports the use of female animals in pre-clinical studies.

In conclusion, our preclinical, in vitro, model of paclitaxel-evoked CIPN provided insights of a direct drug effect on the electrogenic activity of IB4(−) and IB4(+) neurons. Our findings substantiate the use of pre-clinical in vitro models to investigate the direct effect of drugs on neural excitability, the underlying mechanisms and to unveil sex differences. In vitro models have the advantage of simplicity, compared with in vivo animal models that necessarily involve the contribution of different cell types. However, as a trade-off, in vitro models for the highly heterogenous peripheral sensory system may favour cellular populations such as small sensory neurons or myelinated neurons depending on the trophic factors used for maturation (Höke et al., 2003). Nonetheless, models in a dish pave the way for using in vitro human sensory neurons reprogramed from iPSC or transdifferentiated from fibroblasts (Blanchard et al., 2015; Chambers et al., 2012) as preclinical models to investigate the pathophysiology of peripheral neuropathies and to test drug candidates.

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AUTHOR CONTRIBUTIONS

EVR designed and performed the experiments, analysed the data, prepared the figures and revised the manuscript; RTM analysed the data, prepare and revised the figures and revised the manuscript; AFC revised the figures and manuscript; AFM revised the figures and data analysis, designed the experiments and wrote the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Supplementary Material

Table S1. Time course of paclitaxel effect on electrogenic activity of sensory neurons.

	0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
	Vehicle	Paclitaxel	Vehicle	Paclitaxel	Vehicle	Paclitaxel
SA (%) n_{SA} /n_{total}	24 8/34	33 10/30	22 10/46	52* 28/54	22 11/50	36 18/50
Rheobase (pA)	60 (30-103)	80 (28-130)	60 (30-100)	30* (10-50)	50 (20-80)	40 (20-90)
Tonic firing (%) n_{tonic} /n_{total}	44 15/34	57 17/30	46 21/46	81* 44/54	44 22/50	66* 33/50

The percentage of neurons with spontaneous activity (SA), rheobase and percentage of neurons with tonic firing at 0 h (DIV 6), 48 h (DIV 8) and 96 h (DIV 10) post-treatment are depicted. Vehicle and Paclitaxel were compared using the Mann-Whitney test for rheobase, and the Fisher's exact test for SA and Tonic firing. *N* = 15, *n* = 34 for vehicle and *n* = 54 for paclitaxel 24 h after exposure; *N* = 20, *n* = 46 for vehicle and *n* = 54 for paclitaxel, 48 h after exposure; *N* = 19, *n* = 50, 96 h after exposure. ***P* < 0.05 significantly different from vehicle, and values highlighted in orange.

Table S2 - Electrical activity of IB4(-) neurons after vehicle or paclitaxel exposure.

IB4 (-)						
	0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
	Vehicle	Paclitaxel	Vehicle	Paclitaxel	Vehicle	Paclitaxel
n	21	21	49	42	25	25
RMP (mV)	-53 ± 5	-52 ± 6	-52 ± 5	-48 ± 5*	-53 ± 5	-52 ± 5
C (pF)	15 (11-22)	14 (12-21)	16 (13-24)	15 (12-18)	17 (14-25)	17 (15-21)
R_{input} (MΩ)	533 ± 243	399 ± 144*	424 ± 181	491 ± 170	403 ± 183	319 ± 108
SA (%)	43	57	31	67*	40	52
SA f (Hz)	1.6 ± 1.6	2.5 ± 3.1	1.3 ± 1.5	0.9 ± 0.9	1.2 ± 0.9	1.8 ± 1.8
AUC (Hz • pA)	3585 (548-5185)	3955 (1690-5909)	1108 (233-3368)	3330* (1470-5335)	2213 (808-6305)	5130 (1993-6873)
Rheobase (pA)	30 (25-65)	20 (10-70)	40 (20-100)	10 (5-30)*	30 (10-55)	40 (20-55)
Tonic firing (%)	71	95*	55	95*	68	84

Table indicating the number of recordings for each group (n), resting membrane potential (RMP), capacitance (C), percentage of neurons with spontaneous activity (SA), spontaneous activity frequency (SA f), input resistance (R_{input}), Area under the curve of the firing frequency as a function of the current injected (AUC), rheobase, and percentage of neurons with tonic firing (Tonic firing) of IB4(-) exposed to vehicle or paclitaxel for 24 h. Statistical analysis performed between vehicle and paclitaxel groups within each subtype were: Unpaired t-test for RMP, SA f and R_{input}; Mann-Whitney test for C, AUC and rheobase and Fisher's exact test for SA and Tonic firing. Significant differences are indicated in the table with ***P* < 0.05 indicating significantly differences from vehicle and highlighted in orange. *N* = 9, at DIV 6; *N* = 20, at DIV 8; *N* = 10, at DIV10.

Table S3 - Electrical activity IB4(+) neurons after vehicle or paclitaxel exposure.

IB4 (+)						
	0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
	Vehicle	Paclitaxel	Vehicle	Paclitaxel	Vehicle	Paclitaxel
n	17	15	23	27	25	27
RMP (mV)	-51 ± 7	-54 ± 5	-52 ± 4	-50 ± 5	-50 ± 6	-52 ± 4
C (pF)	26 (19-27)	31 (26-37)	23 (19-27)	23 (17-26)	20 (16-27)	20 (16-26)
R_{input} (MΩ)	433 ± 195	355 ± 122	406 ± 165	403 ± 136	437 ± 241	473 ± 252
SA (%)	6	7	0	37*	4	19
SA f (Hz)	0.5	1.0	-	0.8 ± 0.7	0.8	1.0 ± 0.9
AUC (Hz • pA)	235 (130-455)	185 (165-265)	215 (195-1090)	1600 (275-4060)*	255 (205-1583)	675 (215-3300)
Rheobase (pA)	70 (60-175)	120 (80-140)	90 (50-100)	30 (20-70)*	70 (50-100)	70 (30-100)
Tonic firing (%)	12	20	30	63*	12	44

Table similar to Table 2 obtained for IB4(+) neurons. Statistical analysis performed between control and paclitaxel groups within each subtype were: Unpaired t-test for RMP, SA f and R_{input}, Mann-Whitney test for C, AUC and rheobase and Fisher's exact test for SA and Tonic firing. Significant differences from vehicle are indicated in the table with ***P* < 0.05 and highlighted in orange. *N* = 10, at DIV 6; *N* = 13, at DIV 8; *N* = 13, at DIV 10.

Table S4 - Difference on the neuronal electrical activity after paclitaxel exposure between male and female IB4(-) and IB4(+) DRG neurons.

	IB4(-)				IB4(+)			
	Male		Female		Male		Female	
	Vehicle	Paclitaxel	Vehicle	Paclitaxel	Vehicle	Paclitaxel	Vehicle	Paclitaxel
n	23	19	26	23	10	10	13	17
RMP (mV)	-51 ± 5	-48 ± 6	-52 ± 6	-48 ± 5*	-50 ± 4	-51 ± 5	-53 ± 5	-49 ± 4
C (pF)	14 (12-24)	16 (12-20)	18 (13-24)	15 (12-15)	21 (16-27)	23 (20-28)	23 (19-26)	21 (16-26)
SA (%)	26	63*	35	70*	0	40	0	35*
SA f (Hz)	1.3 ± 2	0.7 ± 0.8	1.3 ± 1.4	1.1 ± 1.0	–	1.1 ± 0.8	–	0.6 ± 0.6
Tonic (%)	52	95*	58	96*	40	60	23	65
AUC (Hz • pA)	1815 (260-4135)	3332 (1418-5783)	613 (209-3406)	3300 (1930-5348)*	603 (213-3794)	1348 (255-4026)	205 (180-273)	1600 (300-4620)*
Rheobase (pA)	30 (20-95)	20 (10-35)	40 (20-113)	20 (5-40)*	70 (35-100)	90 (80-120)	50 (28-70)	30 (5-55)*

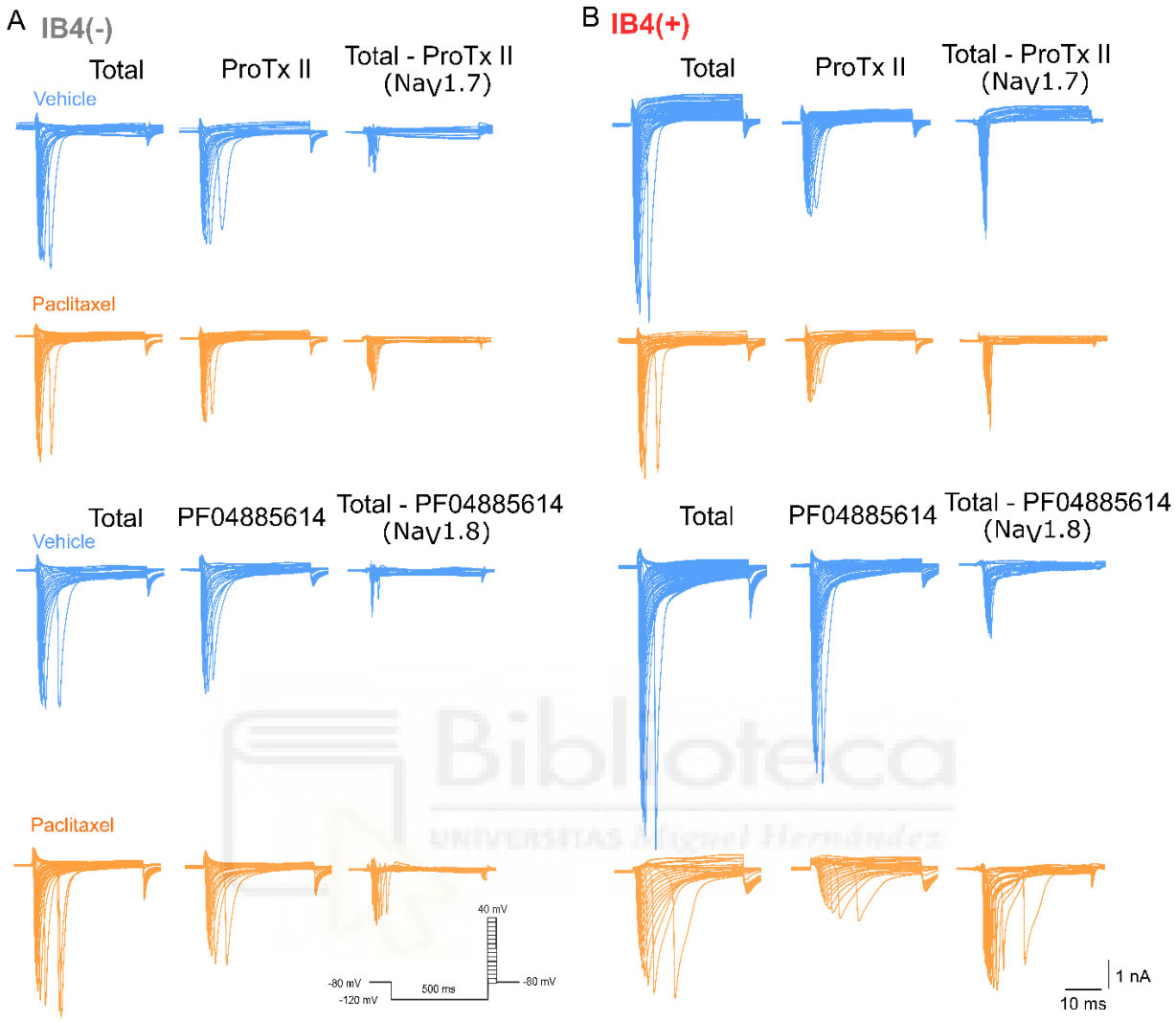
Indicated parameters are: n: number of cells registered, RMP: resting membrane potential, C: capacitance, SA: percentage of neurons with spontaneous activity, SA f: frequency of spontaneous activity, Tonic firing: percentage of neurons with tonic firing, AUC: area under curve of the firing frequency against the current injected, and Rheobase. *N* = 9 for male IB4(-); *N* = 11 for female IB4(-); *N* = 6, for male IB4(+); *N* = 7, for female IB4(+). Vehicle and paclitaxel-treated groups were compared with two-way ANOVA with Tukey's multiple comparisons for RMP; Kruskal-Wallis with Dunn's multiple comparisons for rheobase and AUC; Fisher's exact test for SA (%) and Tonic firing (%). Significant differences from vehicle are indicated in the table with ***P* < 0.05 and highlighted in orange.

Table S5. mRNA expression levels of different ion channels 48 h after vehicle or paclitaxel exposure.

	mRNA expression ($x10^{-3}$)		
Ion channel	Vehicle	Paclitaxel	N
TRPV1	10 ± 3.9	8.3 ± 3.6	4
TRPA1	39 ± 22	39 ± 27	4
TRPM8	20 ± 25	47 ± 78	5
$Na_V1.7$	1.8 ± 0.6	2.8 ± 3.6	6
$Na_V1.8$	0.8 ± 0.3	0.5 ± 0.2	3
$Na_V1.9$	1.2 ± 0.7	1.5 ± 1.3	6

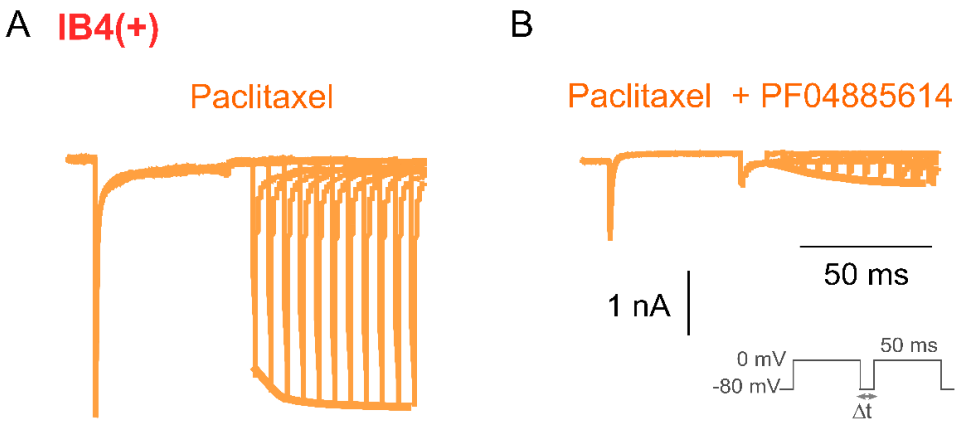
mRNA levels of the indicated ion channels 48 h after vehicle or paclitaxel exposure. mRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method normalizing their expression with the levels of the endogenous control Rpl29. *N*: number of independent cultures analyzed. Values are expressed as mean ± SD.

Figure S1



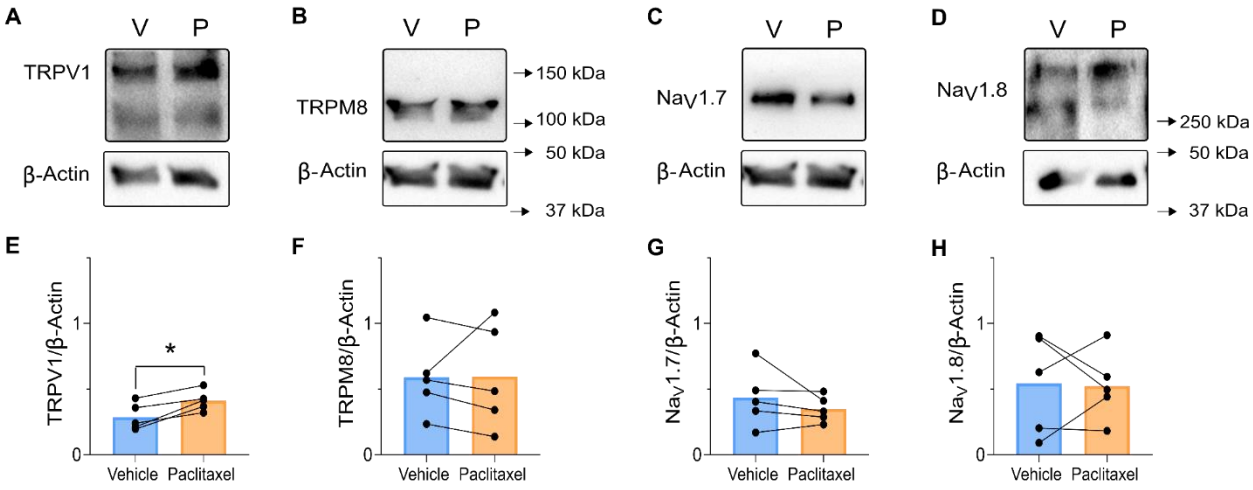
Supplementary Figure 1. Representative Na_V current traces recorded with application of the blockers ProTx II and PF04885614 on IB4(-) and IB4(+) neurons. Current traces evoked with the voltage clamp steps pulses shown in Figure 6 before and after application of ProTx II (10 nM) and PF04885614 (75 nM) with the subtraction procedure performed for isolating $Na_V1.7$ and $Na_V1.8$ currents for IB4(-) (**A**) and IB4(+) (**B**) neurons. Data were collected 48 h post-treatment.

Figure S2



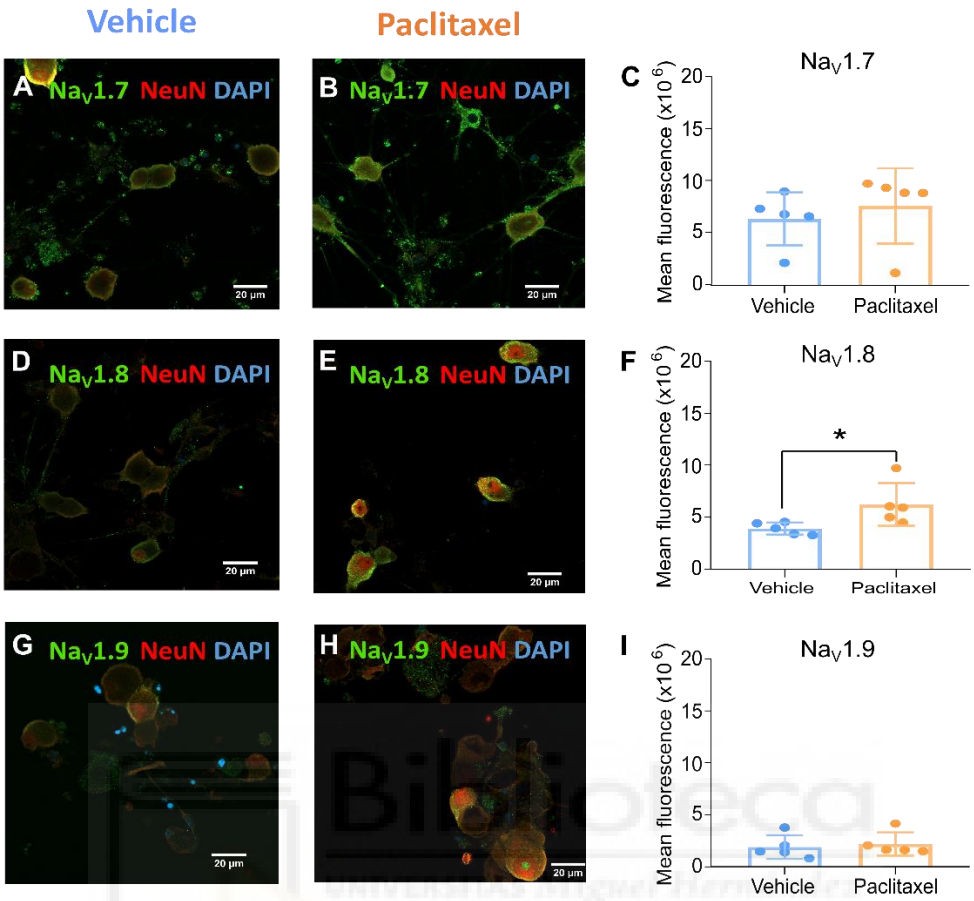
Supplementary Figure 2. PF04885614 inhibited Na_V recovery from inactivation. Representative registers of the Na_V recovery from inactivation before (**A**) and after treatment with the $Na_V1.8$ specific blocker PF04885614 at 75 nM (**B**) for IB4(+) neurons treated with paclitaxel.

Figure S3



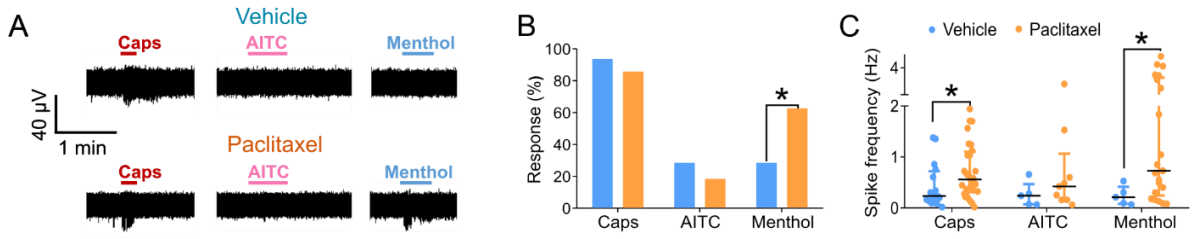
Supplementary Figure 3. TRPV1 protein expression is increased 48 h after paclitaxel exposure. A-D) Representative western blotting for the protein levels of TRPV1 (**A**), TRPM8 (**B**), $Na_V1.7$ (**C**), $Na_V1.8$ (**D**) 48 h after vehicle (V) or paclitaxel exposure (P). Position of the molecular weight marker bands is the same for figures A and B (middle) and for C and D (right). **E-H**) Relative protein levels measured for each channel. β-actin was used as an internal control and the intensity of each band was normalized respect to this protein. Bars indicating mean and individual values represented as dots. Vehicle and paclitaxel measurements from the same culture are connected with lines. $*P < 0.05$ between vehicle and paclitaxel TRPV1 levels. Paired t-test. $N = 5$.

Figure S4



Supplementary Figure 4. Paclitaxel increased Nav1.8 immunoreactivity in sensory neurons. Immunocytochemical staining of cultured DRG neurons treated with vehicle or paclitaxel using anti-Nav1.7, anti-Nav1.8 or anti-Nav1.9 antibodies along with the neuronal marker NeuN and DAPI staining. Green: Nav1.7 (**A, B**), Nav1.8 (**D, E**) or Nav1.9 (**G, H**). Red: NeuN. DAPI: cyan. Scale bar denotes 20 µm. (**C, F and I**) Mean fluorescence intensity of Nav1.7 (**C**), Nav1.8 (**F**) or Nav1.9 (**I**) immunoreactivity for vehicle and paclitaxel treated neurons. Dots correspond to the mean values measured for each independent culture and lines represent the mean ± SD. Data were collected 48 h post-treatment. Statistical analysis was performed using the Unpaired t-test. *N* = 5 independent cultures. ***P* < 0.05, indicating significant statistical differences between vehicle and paclitaxel treated cells.

Figure S5



Supplementary Figure 5. Long-term DRG neuronal cultures show increased TRPV1 and TRPM8 mediated activity after paclitaxel exposure on microelectrode arrays (MEA). (**A**) Representative MEA recordings performed 48 h after vehicle or paclitaxel treatment (DIV 8). The protocol followed consisted of two applications of 20 s of capsaicin (Caps) at 1 µM, application of 50 s of allyl isothiocyanate (AITC) at 100 µM and application of 45 s of menthol at 100 µM. (**B**) Percentage of electrodes with response to Caps, AITC or menthol respect to the total active electrodes. Fisher's exact test. (**C**) Spike frequency of the responses to Caps, AITC and menthol. Dots correspond to the values measured for each electrode and lines represent the median (IQR). Mann-Whitney test. *N* = 3, number of MEA plates = 6. Number of units = 17. ***P* < 0.05 indicating significant statistical differences between vehicle and paclitaxel groups.

Table S6. TRPV1, TRPA1 and TRPM8 activities after paclitaxel exposure.

		0 h (DIV 6)		48 h (DIV 8)		96 h (DIV 10)	
		Vehicle	Paclitaxel	Vehicle	Paclitaxel	Vehicle	Paclitaxel
TRPV1	IB4(-)	16 (8-60)	24 (14-44)	12 (5-30)	48 (23-75)*	10 (6-22)	28 (11-52)
	IB4(+)	5 (2-10)	7 (3-8)	3 (1-9)	9 (1-14)	5 (1-12)	8 (2-23)
TRPA1	IB4(-)	2 (1-3)	1 (1-2)	2 (1-3)	4 (2-4)	2 (1-4)	1 (1-5)
	IB4(+)	4 (2-7)	1 (1-1)	5 (1-12)	4 (3-7)	3 (2-10)	3 (2-6)
TRPM8	IB4(-)	5 (2-8)	6 (4-12)	3 (2-5)	8 (6-12)*	4 (2-8)	9 (3-15)*
	IB4(+)	ND	ND	ND	ND	ND	ND

Current densities for each channel measured at 0 h (DIV 6), 48 h (DIV 8) and 96 h (DIV 10) post-treatment for IB4(-) and IB4(+) DRG neurons expressed in $pA \cdot pF^{-1}$. Vehicle and Paclitaxel were compared using the Mann-Whitney test for TRPV1 and TRPA1, and the Unpaired t-test for TRPM8. Data are expressed as median (Q25-Q75). For TRPV1 and TRPA1 response in IB4(-): $N = 10$, at DIV 6; $N = 11$, at DIV 8; $N = 10$, at DIV10; For TRPM8 response in IB4(-): $N = 5$, at DIV 6; $N = 8$, at DIV 8; $N = 5$, at DIV 10; For TRPV1 and TRPA1 response in IB4(+): $N = 8$, at DIV 6; $N = 11$, at DIV 8 and DIV 10; For TRPM8 response in IB4(+): $N = 3$. Significant statistical differences indicated with $*P < 0.05$, and values highlighted in orange. ND: non-detected.

Annex 2.
Publication 2

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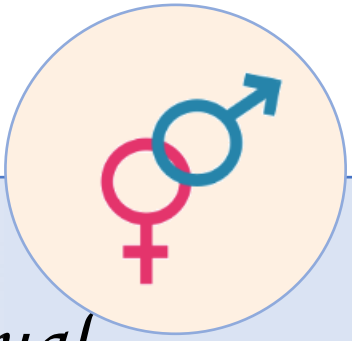
An International Review Journal



ThermoTRP channels in pain sexual dimorphism: new insights for drug intervention

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ThermoTRP channels in pain sexual dimorphism: new insights for drug intervention

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ABSTRACT

Chronic pain is a major burden for the society and remains more prevalent and severe in females. The presence of chronic pain is linked to persistent alterations in the peripheral and the central nervous system. One of the main types of peripheral pain transducers are the transient receptor potential channels (TRP), also known as thermoTRP channels, which intervene in the perception of hot and cold external stimuli. These channels, and especially TRPV1, TRPA1 and TRPM8, have been subjected to profound investigation because of their role as thermosensors and also because of their implication in acute and chronic pain. Surprisingly, their sensitivity to endogenous signaling has been far less studied. Cumulative evidence suggests that the function of these channels may be differently modulated in males and females, in part through sexual hormones, and this could constitute a significant contributor to the sex differences in chronic pain. Here, we review the exciting advances in thermoTRP pharmacology for males and females in two paradigmatic types of chronic pain with a strong peripheral component: chronic migraine and chemotherapy-induced peripheral neuropathy (CIPN). The possibilities of peripheral druggability offered by these channels and the differential exploitation for men and women represent a development opportunity that will lead to a significant increment of the armamentarium of analgesic medicines for personalized chronic pain treatment.

Contents

Abbreviations: 5-HTF1, 5-hydroxytryptamine 1F; ASCO, American Society of Clinical Oncology; CGRP, calcitonin gene related peptide; CIPN, chemotherapy induced peripheral neuropathy; CLR/RAMP1, calcitonin receptor-like receptor/receptor activity modifying protein-1; DRG, dorsal root ganglion; eGFP, enhanced green fluorescent protein; ER, estrogen receptor; FDA, Food and Drug Administration; IB4, Isolectin B4; iPSC, induced pluripotent stem cells; NGF, nerve growth factor; NO, nitric oxide; NSAIDs, Nonsteroidal anti-inflammatory drugs; PACAP, pituitary adenylate cyclase-activating polypeptide; PAG, periaqueductal gray; PKCε, protein kinase C-epsilon; ROS, reactive oxygen species; RVM, rostral ventromedial medulla; S1PR1, sphingosine-1-phosphate receptor; σ-1R, sigma-1 receptor; SD, spreading depression; SNP, single-nucleotide polymorphisms; TLR, Toll-like receptor; TNC, trigeminal nucleus caudalis; TrkA, tropomyosin-related kinase A; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPM3, transient receptor potential melastatin 3; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor potential vanilloid type 1; TRPV2, transient receptor potential vanilloid type 2; TRPV3, transient receptor potential vanilloid type 3; TRPV4, transient receptor potential vanilloid type 4.

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1. Introduction

Chronic pain is a major health challenge of our society affecting up to 20% of the population that suffer pain daily. The high prevalence and the disabling condition of pain has a notable social and economic impact. A seminal study estimated an annual yearly cost of 635 billion$ for USA. This estimate includes the main costs associated to the syndrome, namely cost of medical care and treatment and productivity loss (Gaskin & Richard, 2012). Similar values could be estimated for the rest of the world suggesting an overall economic impact of 3–10% GDP (Breivik, Eisenberg, & O'Brien, 2013). Despite this enormous societal burden, the therapeutics for treating chronic pain continue to be surprisingly limited, being still concentrated mainly in nonsteroidal anti-inflammatory drugs (NSAIDs) alone or combined with acetaminophen along with anti-convulsant and anti-epileptic pharmacotherapy for pain types resistant to current analgesics.

Several reasons may be considered for the poor efficacy of the pharmacological armamentarium available to alleviate chronic pain: (i) traditionally, pain has been considered an associated symptom to a disease rather than an illness itself. Fortunately, chronic pain is currently treated as a malady, which is resulting in a better medical and pharmaceutical attention of patients; (ii) chronic pain is a complex, multifactorial disease involving nociceptive, affective and/or cognitive aspects (Raja et al., 2020), which require an integrated and coordinated interdisciplinary collaboration of preclinic and clinic researchers; (iii) the genetic/epigenetic factors driving predisposition to develop chronic pain constitutes still a major gap of our knowledge; (iv) pre-clinical animal models partially mimic the pathophysiology of chronic pain, and for some types of pain such as migraine, back pain and fibromyalgia we do not have yet compelling models; (v) opiates, the cornerstone for the management of acute moderate to severe pain, show poor efficacy in chronic pain and represent a source of adverse events that can threaten the safety of the chronic patients; (vi) pre-clinical testing to evaluate the anti-hyperalgesic and anti-allodynic potency and efficacy of drug candidates has been performed in cohorts of young males, resulting in a plethora of analgesic candidates that failed in clinical trials that use heterogeneous cohorts of patients; and, (vii) chronic pain exhibits a strong sex dimorphism with an overall higher prevalence in women than in men that has been largely overlooked by the preclinical research community until 2014 (Clayton & Collins, 2014). Noteworthy, the pain community has started to consider sex as a significant variable, resulting in the use of females in preclinical studies, including when testing drug candidates. Taken together, a notable endeavor in the right direction has been undertaken, although additional efforts are still required to fully comprehend the underlying mechanisms contributing to the sexual dimorphism of chronic pain.

Transient receptor potential (TRP) channels are sensory proteins that include more than 50 cation channels with widely varying functions and ubiquitous distribution in various cells and tissues. Some members of the TRP channel family, such as ankyrin 1 (TRPA1), vanilloid subtypes 1, 2 and 4 (TRPV1, 2 and 4), and melastatin 3 and 8 (TRPM3 and TRPM8), are sensors of thermal, mechanical and chemical stimuli (Huang, Li, Dhaka, Story, & Cao, 2012; Sousa-Valente, Andreou, Urban, & Nagy, 2014). The physiological relevance of TRP channels was recently acknowledged after the award of the Nobel Prize of Medicine to Prof. David Julius and Ardem Patapoutian, for their discoveries on these thermal and mechanical transducers (Ledford & Callaway, 2021). These channels are abundantly expressed in primary sensory neurons mainly in dorsal root and trigeminal ganglia (Huang et al., 2012; Sousa-Valente et al., 2014). Other less-studied thermosensory channel are TRPV3, primarily expressed in keratinocytes (Peier et al., 2002) and TRPV4, found in primary afferents and skin cells including keratinocytes, macrophages and mast cells (Zhang, Henry, & Chen, 2021). These channels are members of the so-called thermoTRP channel family and encode neuronal responses from noxious cold to harmful heat (Fig. 1). Notably, simultaneous knockout of TRPV1, TRPA1 and TRPM3 eliminated acute noxious heat responses in mice (Vandewauw et al., 2018), while deletion of TRPM8 affected cold sensing (Peier et al., 2002). Complementary, thermoTRPs are also gated by chemical agents such as capsaicin (TRPV1), menthol (TRPM8), allicin (TRPA1) and pregnenolone sulfate (TRPM3) (Silverman, Chen, Kravatz, Chavan, & Chang, 2020; Vriens et al., 2011), and the activity of some members may be notably enhanced by pro-inflammatory and/or pro-algesic agents (Silverman et al., 2020). Because of their Ca^{2+} permeability, stimulation of most thermoTRP channels promotes the release of pro-inflammatory peptides such as αCGRP that contribute to peripheral sensory sensitization (Alarcón-Alarcón et al., 2022; Bautista et al., 2005; Citak et al., 2022; Devesa et al., 2014; Kichko, Neuhuber, Kobal, & Reeh, 2018; Meng et al., 2009; Ponsati et al., 2012), as well as to trigger pain episodes such as those in migraine (Meng et al., 2009). An important progress has been attained understanding some of the physiological roles of these channels, unveiling a contribution to body temperature homeostasis (Señarís, Ordás, Reimúndez, & Viana, 2018), which appears important to prevent the thermal dysfunction produced by potent and selective channel antagonists (Koivisto, Belvisi, Gaudet, & Szallasi, 2022). Structurally, thermoTRP channels exhibit an overall similar structure of four identical subunits, assembled around a central aqueous pore that organizes the permeation pathway (Fig. 1), and includes a selectivity ionic filter and the channel gates (Latorre, Zaelzer, & Brauchi, 2009). Cryo-electron microscopy has significantly contributed to unveil the structure-function correlates of thermoTRPs and paved the way for a rational design of drugs that control dysfunctional channels in pathological states, such as chronic inflammatory pain, chemotherapy-induced peripheral neuropathy (CIPN) and migraine (Artero-Morales, González-Rodríguez, & Ferrer-Montiel, 2018; Schumacher, 2010; Villalba-Riquelme, de la Torre-Martínez, Fernández-Carvajal, & Ferrer-Montiel, 2022).

Noteworthy, a growing body of research suggests the functional modulation of these receptors by sex hormones that may contribute to the different sex prevalence observed in conditions of chronic pain, as exemplified by migraine that exhibits a 3 times higher prevalence in women than in men (Artero-Morales et al., 2018; Stewart, Shechter, & Rasmussen, 1994). For instance, TRPM8 appears to be activated by testosterone (Asuthkar et al., 2015), and to a lower extent also by progesterone and estradiol (Mohandass et al., 2020). Indeed, TRPM8 has shown roles in pain, migraine, CIPN, and dimorphic sexual behavior (Alarcón-Alarcón et al., 2022; Chasman et al., 2011; Liu et al., 2013; Mohandass et al., 2020; Villalba-Riquelme et al., 2022; Wei, Kim, & McKemy, 2022). TRPM3 is gated by pregnenolone sulfate (Vriens et al., 2011), and TRPV1 and TRPA1 appear to interact with the progesterone receptor Sig-1a receptor (Cortés-Montero, Sánchez-Blázquez, Onetti, Merlos, & Garzón, 2019; Marcotti et al., 2022). Thus, modulation of thermoTRPs by sex hormones open new venues for understanding the sex dimorphism in chronic pain, as well as to develop more personalized analgesic drugs.

Sex differences in pain prevalence and severity are quite evident and excellent reviews have been published on sex dimorphism in chronic pain, including both preclinical and clinical studies, and the reader is referred to these reviews for further information (Gregus, Levine,

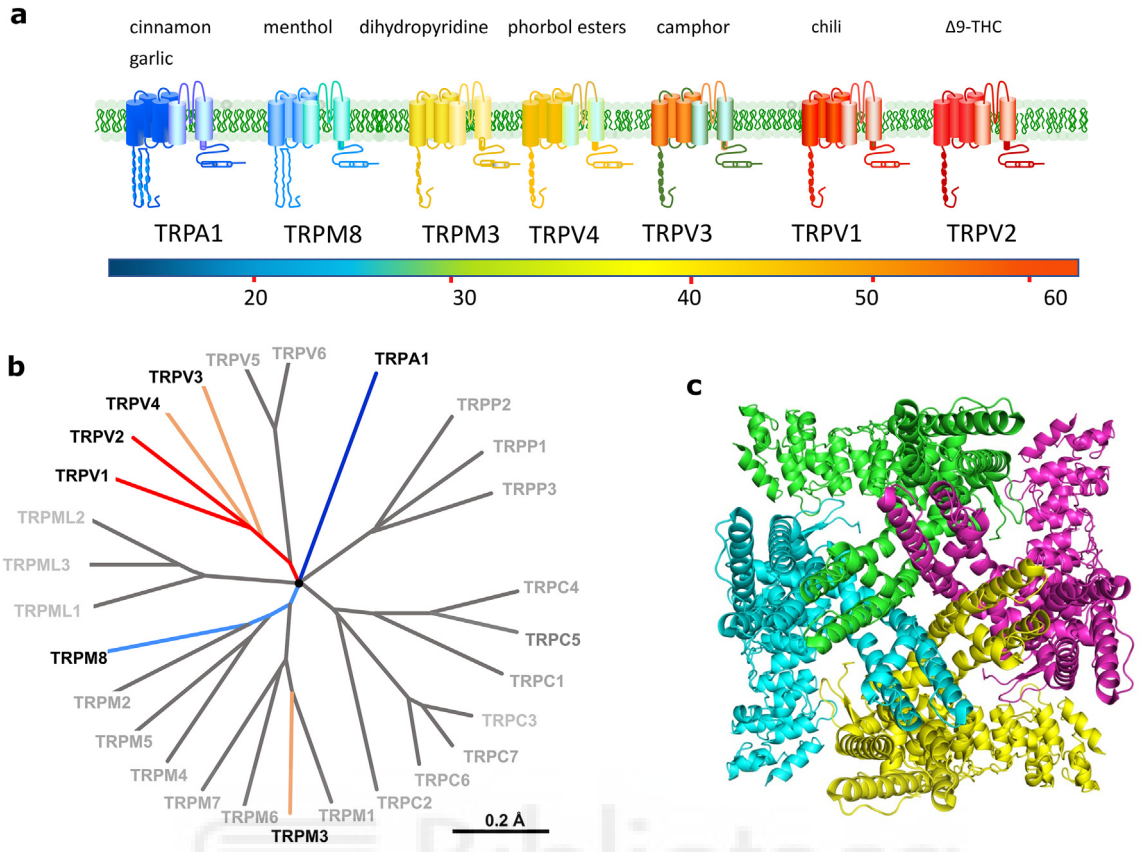

Fig. 1. ThermoTRP Channels. a. Schematic representation of the six mammalian thermoTRP channels. Each subunit consists of six transmembrane domains (S1–S6), a hydrophilic pore loop linking transmembrane segments five (S5) and six (S6), and large cytoplasmic N- and C- terminals (NB: not drawn to scale). All thermoTRPs have a variable number of ankyrin repeat domains in the N-terminus (except TRPM8 which has none). ThermoTRPs display distinct thermal thresholds from noxious hot (TRPV2) to cold (TRPA1). Each thermoTRP is also activated by specific natural compounds and by synthetic substances, which are also known to induce relevant thermal and pain sensations in humans. **b.** Phylogenic tree of human TRP channels. Based on sequence homology, all TRP channels fall into seven subfamilies that comprise proteins with distinct channel properties. The thermoTRP are represented by colors based on their temperature activation range, in deep red appear those channels activated by noxious heat (TRPV2 ≥52°C; TRPV1 ≥43°C), in orange those activated by warm temperatures (TRPV3 ≥36°C; TRPM3 ≥30°C and TRPV4 ≥34°C) in light blue channel activated by cold (TRPM8 ≤28°C), and the one activated by noxious cold (TRPA1 ≤17°C) is represented by deep blue. The bar (0.2) indicates point accepted mutation units, which is the evolutionary distance between two amino acids (1 point accepted mutation unit = 1 point mutation event per 100 amino acids, which is accepted and is passed to progeny). **c.** Upper view of the ribbon structural model of a typical tetramer arrangement of TRP channels. These channels may form homomeric or, within a subfamily, heteromeric channel complexes.

Eddinger, Yaksh, & Buczynski, 2021; Mogil, 2020; Presto, Mazzitelli, Junell, Griffin, & Neugebauer, 2022). These differences appear to have a strong hormonal component, although the precise details and impact are yet under intense investigation. A paradigmatic example of chronic pain syndrome with a significant sex dimorphism is chronic migraine that is up to 3 times more common in women than in men (Lagman-Bartolome & Lay, 2019; Safiri et al., 2022). In addition, chronic migraine also exhibits a strong hormonal influence as the higher prevalence is centered during the women fertile period (Stovner et al. 2018). Similarly, CIPN is a neuropathic type of pain produced due to the nerve damage caused by chemotherapeutic agents that has been also shown to exhibit sex dimorphism (Schmetzer & Flörcken, 2012). For tumors with a similar incidence for women and men, sex differences in CIPN prevalence depends on the chemotherapeutic regime used. Here, we review the information available for the contribution of thermoTRP channels to chronic migraine and CIPN, as these are two paradigmatic chronic pain syndromes that exhibit a demonstrated sex dimorphism. Interestingly, although the molecular mechanisms that cause both pathologies are radically different, they share as a common factor the functional alteration of some TRP channels, the so-called thermoTRP, whose activity is also differently modulated depending on sex. Although still quite in its infancy, the role of sex and gender in chronic pain and its influence in pain therapeutics has become a hot topic in pain transduction and pharmacology research. In this context, thermoTRP receptors have emerged as druggable targets for customized pain control in individuals of any sex.

2. Sexual dimorphism of chronic migraine

Migraine is a recurrent primary head pain of moderate to severe intensity, usually manifested by episodes of disabling unilateral headache with pulsatile characteristics (Ferrari et al., 2022). This clinical condition affects 1 in 7 people, which makes around 1 billion people worldwide (Safiri et al., 2022). Several factors are associated with increased migraine risk, including female sex, being of middle age or overexposure to stressful conditions. Point prevalence of migraine is similar in boys and girls before puberty (~5%) but during adolescence it begins to increase at a higher rate in females, reaching peak levels at around the 40 years of age, when it affects 35% of women and 15% of men. Then, prevalence starts to decrease gradually to become again similar in men and women after the 50 years of age (~5%) (Stovner et al. 2018). Overall, around 2 in 3 migraine patients are females, and compared to men, women also report stronger migraine-related symptoms and disability (Lagman-Bartolome & Lay, 2019). Migraine has a strong polygenetic component (Hautakangas et al., 2022), being highly heritable (34–57%, (Mulder et al., 2003)) and more common in individuals with European ancestors (Key et al., 2018). It has intermediate prevalence in Asian countries located at intermediate latitudes and is less frequent in African populations (Key et al., 2018). The striking higher prevalence of migraine in females has been attributed to genetic factors, to the sexual hormone milieu and to differences in pain and stress responsiveness (Safiri et al., 2022), but the precise mechanisms involved are still poorly understood.

Migraines typically start with prodromal symptoms that last between hours and days and can include fatigue, drowsiness, nausea and vomiting, yawning, mental slowness, impaired concentration, or photophobia (Stovner et al. 2018). This long-lasting premonition is occasionally followed by a minutes-to-hour phase of aura that involves positive visual manifestations such as flashing lights (scintillation) and signs of loss of function like darkening of the visual field (scotoma), paresthesia or motor/speech impairments. Afterwards, the headache crisis begins, and a disabling, generally unilateral and throbbing head pain is presented during 4 to 72 hours, accompanied by photophobia, phonophobia, hypersensitivity to touch and/or nausea and vomiting. The painful and invalidating crisis can be followed by postdromal symptoms which last from hours to days after the resolution of the headache, mainly tiredness, hyperesthesia or allodynia, somnolence, and certain degree of cognitive dysfunction (Ferrari et al., 2022; Louter et al., 2013). In addition, psychiatric and non-psychiatric comorbidities such as anxiety and depression, epilepsy or myocardial infarction can coexist (Ferrari et al., 2022) and the quality of life of the patients is certainly impaired.

NSAIDs or paracetamol constitute the first-line pharmacological treatment for migraine, followed by triptans as second-line medications, alone or combined with fast-acting NSAIDs (Eigenbrodt et al., 2021). Third-line drugs include gepants, which are calcitonin gene related peptide receptor (αCGRP) antagonists and diptans, which are agonists of serotonin 5-HT1F receptors. When required, adjunct prokinetic/antiemetic medications like domperidone or metoclopramide are administered to inhibit nausea and vomiting. Among novel therapies that have been tested to mitigate highly frequent migraine crisis (>15 episodes month) stands up botulinum neurotoxin A (Botox), the first therapeutic specifically approved for chronic migraine by the US Food and Drug Administration (FDA) (Chen et al., 2021), useful for very resistant and disabling migraines. Furthermore, a monoclonal antibody, Erenumab (Aimovig®, Amgen & Novartis), that targets the calcitonin-gen related peptide (αCGRP) receptor, along with Fremanezumab (Ajovy™, TEVA Pharmaceuticals), galcanezumab (Emgality, Eli Lilly) and eptinezumab (Vyepti, Lundbeck), monoclonal antibodies raised against CGRP peptides, were approved as preventive therapy for episodic migraines in 2018 and there is moderate-to-high quality evidence of efficacy for chronic and episodic migraine (Sacco et al., 2022). Note that these therapeutics are targeting the αCGRP signalling pathway, as the "gepant" family of compounds, indicating that this pathway is pivotal in migraine. Noteworthy, αCGRP is released from peptidergic trigeminal nociceptors by activation of thermoTRP channels such as TRPV1 and TRPA1 (Alarcón-Alarcón et al., 2022; Bautista et al., 2005; Devesa et al., 2014; Meng et al., 2009; Ponsati et al., 2012), and modulated by TRPM8 (Citak et al., 2022; Kichko et al., 2018). These channels configurate an interesting therapeutic axis of the αCGRP signalling pathway and may be also involved in the sex dimorphism of the disease.

The period lived with disability in individuals suffering of migraine has kept similar along the years despite the available pharmacotherapy, and the pronounced sexual dimorphism is still patent (Safiri et al., 2022). Thus, the precise causes of the pronounced sexual dimorphism need to be clarified, particularly which pathophysiological differences between males and females are involved and also whether these differences contribute to a divergence in the response to current or potential pharmacological treatments (Paige et al., 2022). In this sense, thermoTRP receptors that have been closely involved in migraine pathophysiology, are abundantly expressed in the trigeminal system innervating the meninges, and, importantly, have shown differential modulation by male and female sexual hormones, thereby they are postulated in this review as potential modulators of chronic migraine amenable for adaptation to men and women.

The pathogenesis of migraine, i.e. the mechanisms explaining why and how migraine crisis are triggered or happen spontaneously, remains poorly understood probably because of the relative unpredictability of migraine attacks (Ferrari et al., 2022). Exposures to environmental irritants or to stressful events are known to trigger migraine-related pain in experimental models and in sensitive individuals, involving the stimulation of primary afferents and the participation of the hypothalamus, a brain region crucial in homeostatic and hormonal control (Iyengar, Johnson, Ossipov, & Aurora, 2019). The aura phase, found in certain individuals, is associated with spreading depression (SD), a slowly-propagating wave of intense depolarization that happens all at once in most neurons and glia of a given gray matter brain region (Ferrari et al., 2022). Such depolarization is followed by suppression of all spontaneous o evoked electrical activity in the same area (depression), generating a wave that spreads at a speed of ~3 mm/s, generally from the occipital area towards more rostral regions (Ferrari et al., 2022). The SD event lasts around 1 minute and is followed by arterial dilation and hyperoxygenation (Ayata & Lauritzen, 2015). At this point, increases in the production/release of Nitric Oxide (NO) and ROS are detected experimentally around trigeminal axons innervating meningeal vasculature (Garthwaite, Charles, & Chess-Williams, 1988; Pradhan, Bertels, & Akerman, 2018; Read, Smith, Hunter, & Parsons, 1997). NO has vasodilatory effects and stimulates neurotransmitter release, especially of αCGRP, a pivotal contributor to migraine pain (Ashina et al., 2019). Other mediators are also released and contribute to neurogenic inflammation and mastocyte degranulation, including substance P, pituitary adenylate cyclase-activating polypeptide (PACAP), bradykinin, neurokinin A, Nerve Growth Factor (NGF), prostaglandin or eicosanoids (Sarchielli et al., 2006; Spekker, Tanaka, Szabó, & Vécsei, 2021). These primary afferent neurons innervating the meninges extend their central terminals to the trigeminal nucleus caudalis (TNC) of the cervical spinal cord, where they synapse with high-threshold and wide dynamic range neurons that receive also input from primary afferents innervating the periorbital skin and pericranial muscles (Ferrari et al., 2022; Iyengar et al., 2019). TNC neurons connect with the thalamus, the first and main relay center of nociceptive information in the brain. There, thalamic neurons integrate peripheral nociceptive information through projections to the somatosensory cortex, which processes information on somatotopic localization, and to limbic regions of the brain such as the amygdala or the striatum, which evoke the affective-motivational components of pain (Bushnell, Ceko, & Low, 2013). TNC neurons also send inputs to the hypothalamus via the thalamus, thereby affecting autonomic responses such as the wake/sleep cycle, the nausea or the vomiting. At the same time there is a hypothalamic/thalamic top-down modulation of the periaqueductal gray (PAG), a brain area crucial in the descending modulation of pain. The PAG conveys affective-motivational, hormonal and circadian/homeostatic information and exerts its modulatory activity through is connections with On and Off cells of the rostral ventromedial medulla (RVM), which facilitate (On) or inhibit (Off) nociceptive transmission in the TNC synapses of the cervical spinal cord (Fields, 2004; Holland, 2009). Indeed, progressive failures in descending inhibitory control of the trigeminal system have been described to favor migraine-related pain in laboratory animals (Boyer, Dallel, Artola, & Monconduit, 2014). Thus, the trigeminal system reacts to peripheral stimulation and to central descending modulation, constituting the cornerstone of migraine pain and offering at the same time the possibility of pharmacological control through peripheral targeting.

2.1. TRP channels in the pathophysiology of migraine

During recent years, a growing body of research has elucidated an important participation of TRP channels in the pathophysiology of migraine, mainly involving TRPA1, TRPV1 and TRPM8 channels, all three receptors prominently involved in thermosensation and expressed in primary afferent neurons of trigeminal and dorsal root ganglia. The presence of these receptors in the meningeal circuitry, normally kept at homeothermic temperature, suggests additional functions other than thermosensation, and offers exceptional possibilities for the control of migraine-related pain in a sex-dependent fashion.

2.2. TRPA1 and TRPV1

The relevance of TRPA1 in pain sensation in humans was evidenced with the finding of a gain-of-function mutation in the TRPA1 gene that causes episodes of debilitating pain after fasting or physical stress (Kremeyer et al., 2010). In the same line, several single-nucleotide polymorphisms (SNP) in the TRPV1 gene have been associated with the presence of migraine (Carreño et al., 2012; Yakubova et al., 2021). TRPA1 and TRPV1 are expressed in ~6–20% and 50% of trigeminal afferents, respectively, and virtually all TRPA1 fibers co-express TRPV1 (Fig. 2, diagram in **b**) (Bautista et al., 2005; Huang et al., 2012). Many of these TRPA1/TRPV1 fibers contain the migraine-inducing neuropeptide αCGRP. In these neurons, stimulation of TRPA1/TRPV1 elicits vesicular release of αCGRP, a process that induces migraine-related pain in preclinical models (Iannone, De Logu, Geppetti, & De Cesaris, 2022) and can be inhibited through specific TRP antagonists or by using exocytosis inhibitors (Alarcón-Alarcón et al., 2022; Bautista et al., 2005; Devesa et al., 2014; Meng et al., 2009; Ponsati et al., 2012).The promiscuity of TRPA1 and TRPV1 renders trigeminal neurons innervating the skin, the tongue or the olfactory system receptive to environmental physical stimuli (extreme cold and heat), and to external agents such as mustard oil, capsaicin, allyl isothiocyanate, acrolein, formaldehyde, cigarette smoke, umbellunone, all stimuli reportedly capable of inducing migraine-related pain in sensitive individuals, experimentally and in real life (Fig. 2, mechanisms in **d**) (Andrè et al., 2008; Bautista et al., 2006; Leishman et al., 2017; Nassini et al., 2012). However, TRPA1 and TRPV1 are also sensitive to physiologically relevant endogenous compounds. For instance, TRPV1 is a receptor of endovanilloids like anandamide (N-arachidonoylethanolamine) one of the main endogenous cannabinoids (Fig. 2, mechanisms in **a**) (Ross, 2003), and TRPA1 is targeted by reactive species of oxidative, nitrative or carbonylic stress (Fig. 2, mechanisms in **a**) (Iannone, Nassini, Patacchini, Geppetti, & De Logu, 2022; Marone et al., 2018; Miyake et al., 2016; Miyamoto, Dubin, Petrus, & Patapoutian, 2009; Sullivan et al., 2015).

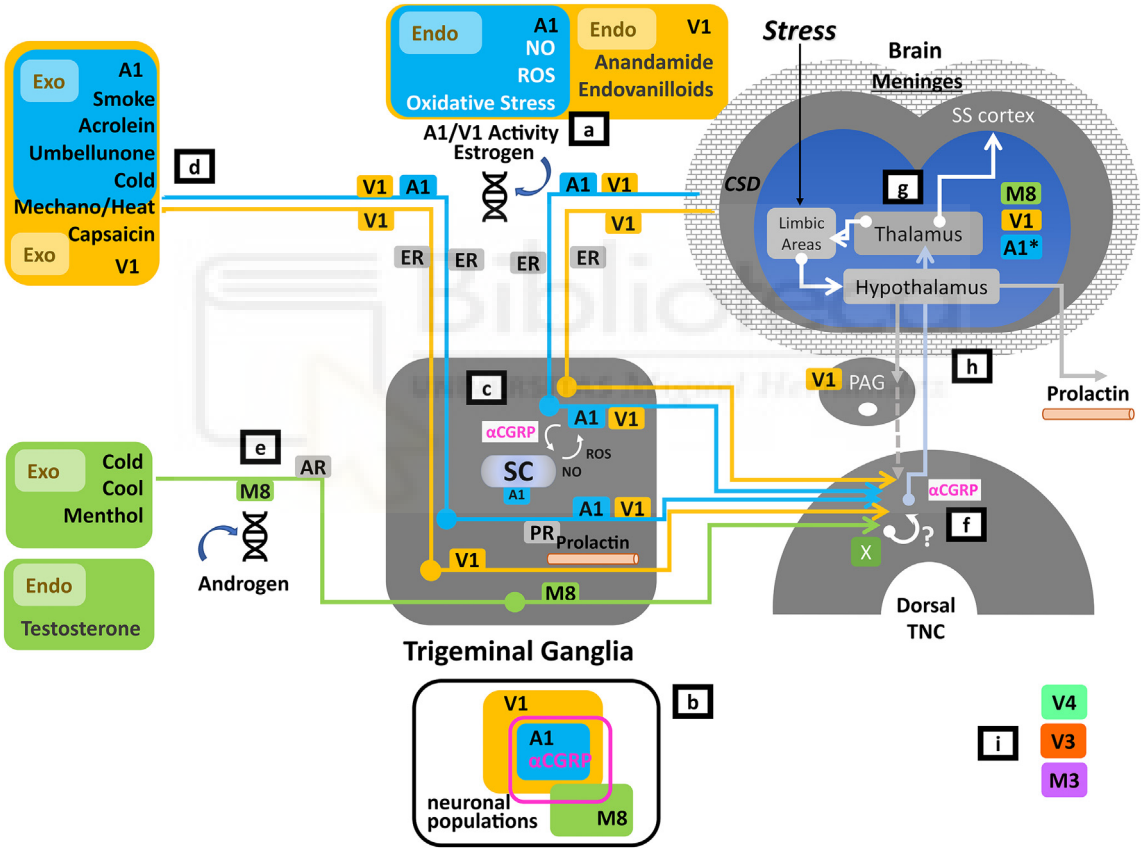

Fig. 2. ThermoTRP channels in migraine and its sexual dimorphism. a. After cortical spreading depression (CSD, Brain) or oxidative stress, endogenous agonists (Endo) of TRPA1 receptor such as Nitric Oxide (NO) or Reactive Oxygen Species (ROS) activate TRPA1 of primary afferents innervating the meninges and promote pain. Endogenous TRPV1 agonists such anandamide or other endovanilloids can also exert pronociceptive effects in the periphery. Stimulation of TRPA1/TRPV1 or estrogenic signaling through the estrogen receptor (ER) facilitates TRPV1 and TRPA1 overexpression. **b.** In the trigeminal ganglia, 97% of TRPA1 and 60–70% of TRPV1 neurons co-express the migraine-inducing neurotransmitter αCGRP, and almost all TRPA1 neurons express also TRPV1. TRPM8 neurons constitute around 10% of trigeminal population of which 26% co-express αCGRP. **c.** TRPA1 / TRPV1 stimulation promotes αCGRP release from trigeminal neurons. αCGRP stimulates receptors in Schwann cells (SC), which in turn release nitric oxide (NO) that activates Schwann cell TRPA1 and promotes generation of reactive oxygen species (ROS). ROS further enhance TRPA1 neuronal activation. Stimulation of prolactin receptor (PR) sensitizes TRPA1/TRPV1 neurons. **d.** Exogenous environmental irritants (Exo) can stimulate peripheral TRPA1/TRPV1 fibers involved in smell, taste, touch, heat, cold or noise detection and facilitate αCGRP release. **e.** Exogenous stimuli (Exo) such as cold/cool temperatures or menthol and the endogenous male hormone testosterone (Endo) can promote TRPM8 activity. Testosterone facilitates TRPM8 expression through stimulation of androgen receptor (AR). TRPM8 stimulation decreases TRPA1/TRPV1-associated pain. **f.** TRPA1/TRPV1 neurons release αCGRP in the dorsal trigeminal nucleus caudalis (TNC) and stimulate 2nd order neurons that send projections to the thalamus. This neurotransmission could be repressed through descending modulation from supraspinal areas or by TRPM8 peripheral activity, either directly through presynaptic glutamate receptors or through interneuron recruitment. **g.** TNC neurons synapse with thalamic neurons that send projections to the somatosensory cortex (SS cortex) which provides information of intensity, quality and duration of pain. Thalamic neurons also project to limbic areas such as the amygdala, which provide the emotional value of pain. TRPV1 and TRPM8 have been described in neurons of multiple brain areas and central TRPA1 expression has been described in glial cells. The central role of these receptors in migraine-related pain is mostly unknown. **h.** Stressful stimuli stimulate brain regions related with affective processing and also modify the activity of the hypothalamus. Female hypothalamic neurons activated after stress release more prolactin than male ones, and prolactin released to the bloodstream can sensitize TRPA1/TRPV1 neurons through prolactin receptor. TRPV1 stimulation in the PAG promotes descending inhibition of pain by modulating TNC neurotransmission through the rostral ventromedial medulla. **i.** TRPV4, TRPV3 and TRPM3 have been related to the pathophysiology of migraine and to sexually dimorphic behaviors but their role in migraine is largely unexplored.

TRPA1 has been proven essential for the pro-nociceptive effects of ROS and NO donors also in preclinical models of acute and chronic migraine (Alarcón-Alarcón et al., 2022; De Logu et al., 2022; Marone et al., 2018) and plays a crucial role on NO-induced vasodilation (Sullivan et al., 2015), two fundamental aspects of migraine pathophysiology. In the periphery TRPA1 is expressed in neurons, but has also been described in fibroblasts, inner ear hair cells, Satellite glial cells and in Schwann cells (SC, Fig. 2, mechanism in **c** (Iannone, Nassini, et al., 2022)). Stimulation of CLR/RAMP1 (calcitonin receptor-like receptor/receptor activity modifying protein-1) in Schwann cells promotes cAMP-dependent NO production, which in turn activates Schwann cell TRPA1 and allows the release of ROS that sustain peripheral pain sensitization through the stimulation of neuronal TRPA1 (De Logu et al., 2022). This mechanism implies possible sensitization of neighboring cells within trigeminal structures, not necessarily affected by the primary sensitization. Thus, the induction of TRPA1/TRPV1-mediated pain through external stimulation of gustatory/olfactory/auditory/skin-innervating primary afferents could trigger sensitization of meningeal neurons through feed-forward stimulation between peripheral glial cells and neurons. Although, the synaptic confluence of first order neurons in common high-threshold or wide-dynamic range 2nd order neurons that may extend their receptive field in the TNC was also described as a central mechanism for this cross-sensory sensitization (Fig. 2, pathway in **f**), (Burstein, Yamamura, Malick, & Strassman, 1998).

Spatial transcriptomics in human primary afferents has recently revealed that women express higher number of differentially-regulated genes in TRPA1-containing fibers than men (Tavares-Ferreira et al., 2022), suggesting basal differences in sensitivity to external and internal stimuli activating TRPA1. This is consistent with the higher peripheral expression of neuronal αCGRP also described in women (Tavares-Ferreira et al., 2022) and endorsed by the high degree of co-localization between TRPA1 and αCGRP in rodents (~97%) (Story et al., 2003). These cellular differences between males and females may be exacerbated after repeated or strong stimulation of peripheral TRPA1 which promote activity-dependent TRPA1 overexpression in preclinical models of pain including migraine (Fig. 2, mechanism in **a** (Alarcón-Alarcón et al., 2022; Martínez-Rojas et al., 2018; McNamara et al., 2007)). While TRPA1 expression or intrinsic functioning seems similar between males and females and its deletion equally abrogates migraine-related pain in both sexes in mice (Alarcón-Alarcón et al., 2022), several basic research studies have described sexual-dimorphic mechanisms that converge on TRPA1-expressing neurons. A recent study has revealed that repeated stressful stimuli induces a specific release of prolactin from hypothalamic arcuate nucleus neurons in females. The subsequent increase in circulating prolactin is able of sensitizing trigeminal TRPA1-expressing neurons through their prolactin receptors, and this priming leads to exaggerated trigeminal painful responses to otherwise innocuous concentrations of the TRPA1 agonist umbellunone (Fig. 2, mechanism in **h**) (Watanabe et al., 2022). Thus, stress-related prolactin release could favor TRPA1-associated migraines preferentially in females through this mechanism. While prolactin is a female hormone particularly abundant during pregnancy and breastfeeding, and both periods are known by having less frequency of migraine (David, Kling, & Starling, 2014), the role of prolactin promoting pain specifically in females has also been described in preclinical migraine models by several groups (Avona et al., 2021; Ikegami et al., 2022) and may involve a sex-biased TRPA1 or TRPV1 signaling (Fig. 2, mechanism in **c**) (Patil, Ruparel, Henry, & Akopian, 2013).

Additional relevant findings potentially contributing to a sexual dimorphism include possible TRPA1 estrogen-dependent expression and stimulation through estrogen metabolites, which promote primary afferent sensitization and pain-like behaviors specifically in females through TRPA1 (Xie et al., 2022). Interestingly, also the use of aromatase inhibitors that prevent the transformation of testosterone to 17-β-estradiol promoted an exacerbated pain sensation (Fusi et al., 2014). Such strong pain sensitization was found to be associated to the direct stimulation of TRPA1 by this type of electrophilic compounds. Their structural similarities with estrogen-related molecules further support the notion that interactions of female steroids with TRPA1 could be a source of sexual dimorphism and may have an impact on the presentation of menstrual migraines. TRPA1 expression has also been described in brain astrocytes, where its activation reduced the activity of inhibitory synapses (Fig. 2, mechanism in **g**) (Shigetomi, Tong, Kwan, Corey, & Khakh, 2011), however, this finding was controversial and several studies failed to report TRPA1 transcripts within the brain (Duque et al., 2022; Zhang et al., 2014). Nonetheless, the absence of TRPA1 in brain neurons seems clear and suggests a reduced possibility of adverse psychotomimetic events. Hence, TRPA1 inhibition or desensitization represents a potential therapeutic strategy for the prevention and relief of migraine-associated pain (Iannone, Nassini, et al., 2022) that should also cover neuroplasticity events idiosyncratic to the female sex. In this regard, clinical trials have been designed to assess the efficacy of selective TRPA1 antagonists inhibiting heat perception (clinicaltrials.gov, NCT05275751) or alleviating neuropathic or acute post-surgical pain (reviewed in Iannone, Nassini, et al., 2022; Souza Monteiro de Araujo et al., 2020). Despite the accumulating preclinical evidence, no studies have been published yet assessing the efficacy of TRPA1 antagonism or desensitization against migraine, and none of them explicitly address a possible sex bias.

TRPV1 has also been closely involved in the pathophysiology of migraine and its targeting through botulinum toxins (Burstein, Blumenfeld, Silberstein, Manack Adams, & Brin, 2020; Pasierski & Szulczyk, 2022) or its peripheral desensitization through specific agonists have shown certain efficacy in alleviating migraine (Fernández-Carvajal, González-Muñiz, Fernández-Ballester, & Ferrer-Montiel, 2020; Saper et al., 2002). In the case of the TRPV1 agonists, adverse effects such as transient hypothermia limited their use. Around 60–70% of TRPV1+ trigeminal neurons co-express αCGRP in male rats (Fig. 2, diagram in **b**) (Price & Flores, 2007) and similar to TRPA1, TRPV1 inhibition decreases αCGRP release from primary afferents (Meng et al., 2009). However, TRPV1 is also found in neurons from brain structures related with pain processing including thalamus, hypothalamus, hippocampus, amygdala or periaqueductal gray (Fig. 2, mechanism in **g**) (Kauer & Gibson, 2009). In these areas, endocannabinoid signaling through TRPV1 can induce pronociceptive (Wu et al., 2010; Xiao et al., 2016) or antinociceptive effects (Barrière et al., 2020; Palazzo, Rossi, & Maione, 2008). For instance, TRPV1 stimulation in the PAG was proven antinociceptive and has been proposed as one of the antinociceptive mechanisms of acetaminophen (Barrière et al., 2020). Furthermore, the reduced fear, anxiety-like behavior or stress-induced sensitization of TRPV1 knockout mice suggests also a role in brain areas implicated in affective-emotional behavior (Kauer & Gibson, 2009). This heterogenous activity indicates a complex role of this receptor at central level, which is in contrast with the clear pronociceptive and vasodilatory actions of peripheral TRPV1 agonism (Caterina et al., 2000; Zygmunt et al., 1999).

Women show stronger trigeminal sensitization than men in response to intradermal application of the TRPV1 agonist capsaicin (Gazerani, Andersen, & Arendt-Nielsen, 2005). This TRPV1-induced sensitization is more intense during the menstrual phase, which also suggests an interplay between female sexual hormones and TRPV1. In addition, estrogen-dependent responses of TRPV1 have been replicated in rodents and cellular models, where female hormones generally favor pain sensitization through TRPV1 (Fig. 2, mechanism in **a,** reviewed in Artero-Morales et al. (2018)). Thus, estrogens have been proposed to modulate TRPV1 activity mainly in three different ways by: (i) genomic regulation where estrogen receptors increase TRPV1 expression through tropomyosin-related kinase A (TrkA) receptors (Artero-Morales et al., 2018; Payrits et al., 2017); (ii) sensitization through intracellular signaling involving PKCε phosphorylation or TrkA receptor activity (Goswami et al., 2011; Gu, Li, & Huang, 2018; Payrits et al., 2017); or, (iii) direct stereospecific interaction where

17-β-estradiol increases open probability of the channel and could have neuroprotective effects (Artero-Morales et al., 2018; Ramírez-Barrantes et al., 2020). Indeed, although the involvement of TRPV1 was not thoroughly explored, the endogenous TRPV1 agonist anandamide produced increased αCGRP release in mesenteric arteries of females but not in males, and this was dependent on 17-β-estradiol that was absent in ovariectomized rats but recovered after supplementation of the hormone (Peroni et al., 2007). Hence, the αCGRP release and vasodilatory effect of anandamide was more prominent in females suggesting a predisposition to vasodilatory effects mediated through TRPV1.

In the same line, anandamide induced central effects selectively in females, impairing fear extinction through signaling at TRPV1 (Morena et al., 2021). Interestingly, modulation of TRPV1 activity has also been observed after exposure to environmental compounds that mimic estrogenic molecules such as bisphenol A (Rossi et al., 2020). This finding is compatible with the exacerbated migraine-like behavior observed after bisphenol-A exposure in a rat model of migraine (Vermeer, Gregory, Winter, McCarson, & Berman, 2014). The sexual dimorphism involving TRPV1 activity in migraine may go beyond its interaction with estrogenic compounds in neurons. For instance, ethanol reduces the activation threshold of TRPV1 and favors its activity at body temperature. In addition, it produces TRPV1 sensitization against the endogenous cannabinoid anandamide (Trevisani et al., 2002). In this scenario, differences in ethanol metabolism or pharmacokinetics between males and females could favor migraine preferentially in women after the intake of alcoholic beverages (Erol & Karpyak, 2015). Another source of migraine modulation specific for women could come from the female hormone progesterone, which binds the endogenous chaperone sigma 1 receptor (σ1R). Since σ1R favors the insertion of functional TRPV1 into the neuronal plasma membrane and progesterone inhibits this process (Ortíz-Rentería et al., 2018), this mechanism could be particularly relevant during pregnancy, a period particularly associated with less incidence of migraines (David et al., 2014). However, none of these latter assumptions have been formally investigated.

The accumulated evidence on the involvement of TRPV1 in migraine yielded a clinical trial assessing the efficacy of the TRPV1 antagonist SB-705498 for the treatment of acute migraine (www.clinicaltrialsregister.eu; EudraCT Number: 2005-004480-37). While there was a lack of benefit when compared to placebo, the brain penetrance described for the antagonist (Lambert et al., 2009) could have promoted mixed antinociceptive and pronociceptive effects. Unfortunately, a clinical trial assessing the efficacy of a peripherally-restricted TRPV1 antagonist for migraine has not been announced yet. The use of topical or peripherally-restricted antagonists or the development of novel photoswitchable agents to modulate TRPV1 and TRPA1 activity through optical stimulation (Frank et al., 2015; Lam et al., 2020; Qiao et al., 2021) may offer new therapeutic opportunities for the inhibition of migraine pain and the disruption of their sexual dimorphic signaling during chronic migraine.

2.3. TRPM8

Multiple genetic studies in humans associate the presence of SNPs affecting the TRPM8 gene with migraine susceptibility (Chasman et al., 2011; Chen et al., 2018; Ling, Chen, Fann, Wang, & Wang, 2019; Siokas et al., 2022). Some of these polymorphisms are linked also with increased allodynia, anxiety and depression in migraine, highlighting the relevance of TRPM8 in its pathophysiology. In this regard, rs10166942 appears associated to an increased risk having the T allele vs the C, (Chasman et al., 2011) or with increased cold sensitivity elicited by the cold pressor test (3–5°C); and, rs7577262 appears associated to an increased risk having the G vs. the A allele (Chasman et al., 2014). Of these polymorphisms, rs10166942 is the most studied one, with carriers of the rs10166942[T] allele having increased risk for migraine and carriers of the rs10166942[C] allele showing protection. Given that the SNP is located upstream to the TRPM8 gene in putative regulatory regions (Key et al., 2018), it could be expected normal functioning of the channel and increased or decreased expression of the protein. In this regard, one report predicted reduced TRPM8 expression in carriers of rs10166942[C] allele and higher TRPM8 levels in rs17862920[T] carriers (Gavva et al., 2019), which also associated with increased cold pain in the cold pressor test. Thus, while the evidence is still weak, increased TRPM8 expression has been interpreted as a possible pain-inducing mechanism absent in rs10166942[C] carriers (Gavva et al., 2019; Wei et al., 2022). Interestingly, the rs17862920[T] allele was also suggested as an evolutionary adaptation to cold temperatures after the observation of a population distribution largely dependent on temperature, higher latitudes and European ancestry (Key et al., 2018), whereas the rs10166942[C] migraine-free allele was found to be more primitive and common in lower and warmer latitudes. Since the SNP affects a genetic region with affinity for a transcription factor, it could be inferred from this study that the reduced cold sensitivity of rs17862920[T] allele carriers could be associated with decreased TRPM8 expression, an evolutive advantage protective against cold that also facilitated migraine (Key et al., 2018). However, it has not been considered whether an overexpressed TRPM8 in individuals with the rs17862920[T] allele could also be an adaptative protective mechanism inherently present in certain migraine populations. In this line, promoting TRPM8 activity through cooling or with canonical TRPM8 agonists such as menthol is a pain-relieving strategy commonly used by migraineurs that has also been used successfully to alleviate migraine pain in clinical studies (Fig. 1, mechanism in **e**) (Borhani Haghighi et al., 2010; Shah et al., 2021; St Cyr et al., 2015).

TRPM8 stimulation has been shown to inhibit TRPA1 and TRPV1-related pain in animal models of migraine (Alarcón-Alarcón et al., 2022; Andersen, Gazerani, & Arendt-Nielsen, 2016; Kayama et al., 2018; Liu et al., 2013; Ren, Dhaka, & Cao, 2015). TRPM8 is expressed in a ~10–13% of trigeminal neurons, a subset of neurons mostly void of TRPA1/TRPV1 channels in laboratory animals and humans (Dhaka, Earley, Watson, & Patapoutian, 2008; Huang et al., 2012; Kobayashi et al., 2005; Story et al., 2003; Tavares-Ferreira et al., 2022) (Fig. 1, diagram in **b**). Although studies using TRPM8 eGFP mice found TRPV1 expression in ~5–20% of TRPM8 fibers (Dhaka et al., 2008; Kobayashi et al., 2005), it is described that dural afferents express TRPM8 receptors in very low proportion (~3–4%, (Huang et al., 2012; Ren et al., 2018)). Of $TRPM8^{+}$ trigeminal neurons, up to 26% were found to express αCGRP (Kim et al., 2014) (Fig. 1, diagram in **b**). The main interactions between TRPM8 and TRPA1/TRPV1 meningeal fibers transmitting migraine-related pain could be expected to occur either at the TNC in the cervical spinal cord or indirectly through higher supraspinal circuitry. This latter possibility builds on the recent description of TRPM8-expressing neurons also in central brain areas, where its function has been scarcely explored (Mohandass et al., 2020; Ordás et al., 2021). Several mechanisms have been proposed to explain the pain-relieving effects of peripheral TRPM8 activity (Fig. 1, mechanism in **f**). It has been described that spinal glutamate release from TRPM8 fibers exerts an inhibitory function through metabotropic glutamatergic receptors expressed either presynaptically in adjacent fibers or post-synaptically (Proudfoot et al., 2006). A cross-inhibitory intracellular effect of TRPM8 over TRPV1-induced c-Jun N-terminal kinase phosphorylation has also been reported (Kayama et al., 2018). In addition, it has been proposed that TRPM8 activation could facilitate recruitment of inhibitory interneurons that silence TRPA1/TRPV1-expressing nociceptors (Dussor & Cao, 2016), although this circuit has not been characterized thus far. Accordingly, TRPM8 activity may occlude signaling of TRPA1/TRPV1-expressing nociceptors and their subsequent αCGRP release.

On the other hand, pronociceptive actions of TRPM8 activity have also been proposed during chronic migraine based on the lower cold pain threshold manifested by a proportion of migraine patients (Nahman-Averbuch et al., 2018) and after the pronociceptive effects observed upon meningeal application of the TRPM8 agonist icilin in rodents (Burgos-Vega et al., 2016). However, sensitivity to extreme cold has been associated also with TRPA1 stimulation and additional

transducers, and icilin is too a known TRPA1 agonist (Buijs & McNaughton, 2020; Paricio-Montesinos et al., 2020; Story et al., 2003; Winter, Gruschwitz, Eger, Touska, & Zimmermann, 2017). Nevertheless, a recent group with extended experience on the pronociceptive actions of TRPM8 has described a pronociceptive function of this receptor also in a model of chronic migraine induced by repeated treatment with the NO donor nitroglycerin containing a percentage of propylene glycol (Wei et al., 2022). According to the authors, TRPA1 could initiate its pronociceptive actions upstream of the TRPM8 fibers (Yamaki, Chau, Gonzales, & McKemy, 2021). Thus, TRPA1-expressing cells would release pronociceptive mediators to sensitize TRPM8 fibers that promote cold sensitivity in migraine. Regardless of the validity of all these assumptions, the accumulating preclinical and clinical evidence underlines the relevance of the TRPM8 receptor in migraine pathophysiology.

An additional effort will be needed to clarify the pronociceptive and/or antinociceptive actions of TRPM8 in preclinical models of migraine and also in the clinics. Available clinical data only describe at most significant pain-relieving efficacy for canonical TRPM8 agonists (Borhani Haghighi et al., 2010; Lopresti, Smith, & Drummond, 2020; St Cyr et al., 2015), however it must be recognized that these agonists lack specificity and can produce desensitization of the channel. Hence, it is unclear whether the obtained outcomes in these studies in humans are due to activation or desensitization. The use of novel specific agonists or additional validation through different types of compounds is still needed to clarify the contribution of TRPM8 to migraine in humans.

TRPM8 expression shows a clear sexual dimorphism in humans, being prominently expressed in male tissues such as the prostate or the testicles (Uhlén et al., 2015). Different genetic studies have revealed trends for increased risk of migraine in female carriers of the TRPM8 SNP rs17862920[T] (Chasman et al., 2011; Ling et al., 2019), although this is in contrast with other studies finding similar prevalence in males and females (Siokas et al., 2022) and with the remarkable sexual dimorphism of general migraine where females represent at least 2 of each 3 patients and develop also more pronounced symptomatology Lagman-Bartolome & Lay, 2019; Stovner et al. (2018). Indeed, one of the genetic studies on the same SNP shows significant association of migraine with the T allele only in males (Kaur, Ali, Ahmad, Pandey, & Singh, 2019), supporting the possibility of TRPM8 being particularly relevant in males. In this line, we have found in our laboratory a dimorphic function of TRPM8 in a mouse model of chronic migraine. The model displays a sexual dimorphic phenotype in which repeated nitroglycerin administration induces persistent hypersensitivity solely in females, whereas males readily recover from the migraine crisis (Alarcón-Alarcón et al., 2022). Using TRPM8 knockout mice, we have found that TRPM8 is essential for the reinstatement of normal sensitivity in males, whereas female mice do not alter their migraine-like behavior (Alarcón-Alarcón et al., 2022). After observing several previous studies suggesting that TRPM8 could act as a testosterone receptor (Asuthkar et al., 2015; Kondrats'kyĭ, Kondrats'ka, Skryma, Prevars'ka, & Shuba Ia, 2009), we tested the hypothesis that testosterone could actually provide protection through TRPM8. In agreement, downregulation of this protective mechanism in males led to persistent mechanical hypersensitivity, whereas acute testosterone favored recovery in females (Alarcón-Alarcón et al., 2022). This pain relief was markedly reduced in TRPM8 knockout mice and was sensitive to the specific antagonist AMTB, although additional TRPM8 antagonists were not assessed. The agonistic activity of physiological picomolar concentrations of testosterone (Yoo & Napoli, 2019) over TRPM8 was corroborated through calcium imaging in cellular models expressing rat and human TRPM8 (Alarcón-Alarcón et al., 2022). Since progesterone and 17-β-estradiol also bind to TRPM8, although with lower affinity than testosterone, their menstrual drop might partially contribute to trigger migraine episodes during menstruation.

Although a previous study suggested that the androgen receptor could exert a tonic inhibition of TRPM8 activity when testosterone is administered at nanomolar concentrations (Gkika et al., 2020), we found that picomolar testosterone-induced currents occurred independently of the expression of canonical forms of the androgen receptor (Alarcón-Alarcón et al., 2022). Hence, we found a testosterone-dependent protective function of TRPM8 (Fig. 1, mechanism in **e**) independent of the androgen receptor, which could explain the acute effects of testosterone treatments (Rosano et al., 1999). This non-transcriptional activity also substantiates fast-acting behavioral effects of testosterone observed just 30 min to one hour after administration, such as the effects on affective behavior found in rodent models (Alarcón-Alarcón et al., 2022; Frye, Edinger, & Sumida, 2008; Mohandass et al., 2020; Rosano et al., 1999). Additional male-specific effects of TRPM8 activity have been evidenced in constitutive knockout mice, with responses to testosterone attenuated in the amygdala and changes in ventral tegmental area that were in correlation with enhanced aggressiveness and reduced sexual satiety (Mohandass et al., 2020). In the same line, aged TRPM8 knockout males develop low bone mineral density as if they were females, whereas the individuals of this sex do not alter their wild-type phenotype after the genetic deletion of TRPM8 (Lelis Carvalho et al., 2021). In the context of persistent pain, androgenic TRPM8 activity may constitute a pain-relieving mechanism present in other models of migraine or chronic pain that also display sexual dimorphism (Lesnak, Inoue, Lima, Rasmussen, & Sluka, 2020; Viero et al., 2022; Watanabe et al., 2022). However, testosterone is a controlled substance that can have deleterious or undesirable effects that prevent its widespread use. The design of TRPM8 agonists that are void of activity over the androgen receptor could represent a potential therapeutic strategy for women or migraine patients with low testosterone levels and may uncover as well additional benefits in light of the results obtained in preclinical models (Lelis Carvalho et al., 2021; Mohandass et al., 2020).

2.4. Other thermoTRP channels

Although most of studies have been focused on the role of TRPA1, TRPV1 and TRPM8 in migraine, there are also relevant findings on less studied thermoTRP channels that could provide insight on migraine sexual dimorphism in the near future (Fig. 1, mechanism in **i**). For instance, TRPV4, a channel activated by hypoosmolarity and membrane stretching, is also expressed in the trigeminal ganglia and shows significantly higher expression in female vs. male mice (Mecklenburg et al., 2020). Interestingly, the lack of TRPV4 increases bone mass and decreases bone elasticity selectively in male mice, and there is a genetic polymorphism linked to fracture risk in men (van der Eerden et al., 2013). The potential relevance of this receptor for migraine was found in a rat model of headache where pain responses increased after dural application of a specific TRPV4 agonist (Wei, Edelmayer, Yan, & Dussor, 2011). Unfortunately, the study was conducted only in males and no sex differences have been reported so far in migraine models. Another genetic study found significant association of migraine with aura with a SNP variant of TRPV3 (Carreño et al., 2012). Remarkably, this thermoTRP modulates the production of NO in the skin independently of nitric oxide synthases and its deletion in mice produces thermosensory deficits selectively expressed in females (Miyamoto, Petrus, Dubin, & Patapoutian, 2011). Finally, the mechano- and thermosensory channel TRPM3 is prominently functional in trigeminal fibers of mouse meninges especially in females (Held, Voets, & Vriens, 2015; Krivoshein, Tolner, Maagdenberg, & Giniatullin, 2022) and is sensitive also to the endogenous neurosteroid pregnenolone sulfate. Its demonstrated participation in pain behaviors in rodent models (Held et al., 2015; Kelemen et al., 2021) suggests the needs of understanding the functionality of this receptor in migraine-related conditions for a complete understanding of migraine sexual dimorphism.

3. Sexual dimorphism in chemotherapy-induced peripheral neuropathy

CIPN is a severe adverse effect produced by cancer chemotherapy. It is a highly prevalent disease occurring in up to 90% of the patients receiving most of chemotherapeutic drugs (Burgess et al., 2021). CIPN

symptoms are characterized by paresthesia, dysesthesia, spontaneous and burning pain, mechanical and thermal hypersensitivity primarily arising in the hands and feet (glove-and-sock distribution) (Burgess et al., 2021). This sensory disorder resolves in 30% of patients after chemotherapy cessation but remains in 40–50% of patients for more than 1 year (Teng, Cohen, Egger, Blinman, & Vardy, 2022). Noteworthy, for some drug regimens a significant ≈30% of patients must stop their cancer treatment because the intense sensory dysfunction produced by CIPN unbearably affects their quality of life, and usually promotes comorbidities such as depression (Prutianu et al., 2022). Furthermore, poor adherence to chemotherapy because of CIPN frequently results in cancer progression, as oncologists are forced to use alternative drugs that are less efficient fighting the tumor. Taking together, CIPN represents an important health problem that affects up to 4% of the population, considering the prevalence of cancer.

Despite its high incidence, there is no effective treatment approved to alleviate CIPN symptoms. Different pharmacological and non-pharmacological interventions have been recommended in several clinical practice guidelines (Jordan et al., 2020; Loprinzi et al., 2020). However, these recommendations come from studies of patients suffering neuropathic pain caused by other diseases rather than CIPN. Among pharmacological interventions, only duloxetine showed a moderate effect in reducing CIPN symptoms of chemotherapy-treated patients and has been recommended in the guidelines of the American Society of Clinical Oncology (ASCO) (Loprinzi et al., 2020; Smith et al., 2013). Since the incidence of this pathology is increasing, there is an urgent need to unveil the underlying mechanisms leading to the peripheral neuropathy, and to develop treatments that increase the quality of life of cancer patients through chemotherapy and promote their adherence to the best anti-tumoral drug regime. Currently, up to 200 clinical trials/studies are registered for testing different strategies for CIPN, although most of them are combination of known drugs or acupuncture methods (https://www.clinicaltrials.gov/ct2/home).

Clinical and pre-clinical studies are providing evidence on the presence of sex dimorphism in CIPN pathophysiology (Davidson et al., 2019; Ferrari, Araldi, Green, & Levine, 2020; Villalba-Riquelme et al., 2022; Wagner et al., 2021). In addition, sex differences in CIPN pharmacology have also been described (Ram et al., 2021), suggesting that adaptation of the therapeutic strategy to the patient sex appears an important factor for a satisfactory disease management. In support of this tenet, promising results were obtained when the chemotherapeutic dose was specifically adapted to each sex and age (Pfreundschuh et al., 2017).

The tight therapeutic window of chemotherapeutic drugs has brought special concerns regarding the existence of sex differences (Wang & Huang, 2007), and different studies are considering sex as a risk factor in the development and magnitude of CIPN (Mizrahi et al., 2021; Sałat, 2020). A summary of reported sex differences in CIPN symptoms for each chemotherapeutic agent is exhibited in Table 1. Among the most common clinical findings, women experience higher incidence, sensitivity and/or severity of peripheral neuropathy for most of the chemotherapeutic regimes (Lévy et al., 1998; Mols et al., 2016; Trendowski et al., 2021; Unger et al., 2022). Nonetheless, a study that analyzed different chemotherapeutic strategies for esophagogastric cancer revealed a greater incidence of CIPN in men for some therapeutic combinations (Davidson et al., 2019). However, a limitation of this study is that the percentage of men and women was not equal in all groups analyzed, which could have influenced the results to certain degree. Other studies such as the work of Yamada et al. (2020) did not find significant differences between sexes in peripheral neuropathy (grade ≥3) following combination of chemotherapeutic treatments. However, differences in baseline characteristics and tumor characteristics of male and female patients might have conditioned the results.

Despite the clinical evidence, the underlying molecular and cellular mechanisms involved in the sex dimorphism of CIPN remain unknown, primarily because most of the pre-clinical research has been performed in male subjects. In addition, CIPN-related sex differences have been less recognized by the scientific community than in migraine (Artero-Morales et al., 2018). Most likely, sex dimorphism in CIPN has been largely overlooked at least in part because it has been considered an adverse effect of cancer therapy and thus unrelated to the disease. Furthermore, identification of sex-driven mechanisms in CIPN has also been challenging because of its complex etiology along with the co-existence of the tumor and the therapeutic strategy associated. In this regard, factors such as tumor metabolism, surgical trauma, immune response, radiotherapy, interference of co-administered drugs, anti-hormonal therapy, and pharmacokinetics and/or pharmacodynamics of the drugs may contribute to CIPN etiology (Wagner, 2020). In addition, the combination of chemotherapeutic drugs adds another dimension to the disorder that further complicate understanding the pathophysiology of the syndrome (Table 1). Noteworthy, previous studies in CIPN incidence have focused on differences in drug pharmacokinetics, tumor biology, survival to cancer and overall drug toxicity (Cristina et al., 2018; Rubin et al., 2020; Sloan et al., 2002; Wagner et al., 2019), but have overlooked the presence of sex differences in CIPN. This lack of information on CIPN sex dimorphism has prevented the development of more adequate therapeutic regimes for women and men. Thus, it is urgent that pre-clinical studies are performed in both sexes to uncover the role of sex in the pathophysiology of CIPN, as this may provide insightful information for more efficient pharmacological approaches.

Recent studies pointed out to a key role of thermoTRP channels on CIPN as well as in the sex dimorphism exhibited by the peripheral neuropathy. The potential molecular mechanisms involved are represented in Fig. 3. In females, the development of mechanical pain in a model of paclitaxel induced-CIPN was described to arise from the IL-23/IL-17A/TRPV1 axis (Luo et al., 2021). This study detected an increase in IL-23 release only in females that was mediated by TRPV1, as it was absent in TRPV1 null mice. Our studies on a preclinical model of paclitaxel-induced nociceptor excitability showed that female sensory neurons exhibited higher spontaneous and evoked excitability than their male counterparts (Villalba-Riquelme et al., 2022). This difference was not apparently mediated by a differential effect of the taxane on TRPV1 functionality as the thermoTRP was similarly affected by the drug in sensory neurons of both sexes, suggesting the involvement of other nociceptor channels. Interestingly, in the same study paclitaxel potentiated TRPM8 activity more intensively in male than in female sensory neurons.

Akin to migraine, modulation of thermoTRP channel function by sexual hormones appears as a potential determinant of the sex dimorphism observed in CIPN. A study by Srinivasan et al. (2008) found that sex hormones play a role in sex differences observed in CIPN by modulating thermoTRP channel function and their signaling pathways. Another study found different levels of gonadal hormones in the peripheral nervous system in male and female rats (Caruso et al., 2013). In particular, testosterone concentration in the sciatic nerve was higher in males whereas 17-β-estradiol and progesterone derivatives (dihydroprogesterone, tetrahydroprogesterone) were increased in females, suggesting that a specific hormonally-activated pathway may be predominating in each sex. An estrogen-dependent sexual dimorphism was also found in rats treated with vincristine, where removal of the hormone abolished the greater mechanical hypersensitivity found in female rats when compared to males (Joseph & Levine, 2003). In this study, inhibition of PKC-ε, a modulator of TRPV1 activity, reduced vincristine-induced hyperalgesia in males and ovariectomized females (Joseph & Levine, 2003), suggesting that estrogens could generate hyperalgesia in females by promoting TRPV1 signaling (Goswami et al., 2011). In support of this tenet, Luo et al. (2021) showed that estrogens activated the IL-23/IL-17A/TRPV1 axis to induce mechanical pain in paclitaxel CIPN. In line with these results, in another paclitaxel model, ovariectomized rats showed an increased threshold to mechanical and thermal stimuli, thus exhibiting a reduced pain response (Wang, Li, Zhao, & Zhang, 2018). Intriguingly, other studies attributed a protective

Table 1
Sex differences found in CIPN pain symptoms after treatment with different chemotherapeutic agents in diverse clinical and/or pre-clinical studies.

Chemotherapeutic Drug	Potential thermoTRPs Involved	Sex differences observed	Reference
Taxanes			
Paclitaxel	TRPV1 TRPM8	Reduced rheobase, higher sensitivity in female rat DRG neurons exposed to paclitaxel	(Villalba-Riquelme et al., 2022)
	TRPV4	Cold allodynia more robust in female mice	(Naji-Esfahani et al., 2016)
	TRPA1	Greater magnitude of paclitaxel hyperalgesia in female rats	(Ferrari et al., 2020)
		Higher TRPM8 expression in male rat DRG neurons	(Villalba-Riquelme et al., 2022)
		Mechanical pain produced through estrogen dependent IL-23/IL-13/TRPV1 signaling axis only in female mice	(Luo et al., 2021)
		No significant sex difference in the response to mechanical stimuli for male and female rats	(Hwang, Kim, Kim, Kwon, & Kim, 2012)
Docetaxel	TRPV1	Not investigated alone	
Platinum-based compounds			
Oxaliplatin	TRPV1 TRPM8 TRPA1	Reduction in nerve conduction amplitude in female mice	(Warncke et al., 2021)
Cisplatin	TRPV1 TRPV2	Tendency in women with advanced non-small cell lung cancer to have more neurosensory deficits than men (clinical trials)	(Wakelee et al., 2006)
	TRPA1	More persistent tactile allodynia in male than in female mice	(Woller, Corr, & Yaksh, 2015)
	TRPM8	Bigger incidence of prolonged heat latency in male rats	(Wongtawatchai, Agthong, Kaewsema, & Chentanez, 2009)
Carboplatin	TRPA1	Not investigated alone	
Vinca-alkaloids			
Vincristine	TRPV1	Mechanical hyperalgesia was higher in female than in male rats	(Joseph & Levine, 2003)
	TRPV4	Statistically significant reduced mechanical sensitivity threshold only in male rats	(Legakis, Diester, Townsend, Karim-Nejad, & Negus, 2020)
Proteasome inhibitor			
Bortezomib	TRPA1 TRPV1	The prevalence of peripheral neuropathy was nearly double in women treated with bortezomib (65.3%) compared to men (36%) (retrospective study using clinical data)	(Martinez et al., 2019)
		Significant lower threshold of mechanical sensitivity at day 30 in female but not in male Sprague-Dawley rats	(Legakis et al., 2020)
		S1PR1 antagonists prevented bortezomib mechano-allodynia and mechano-hyperalgesia in male but not in female rats	(Stockstill et al., 2020)
		Male sex was a predictor of bortezomib-induced CIPN development (retrospective analysis using clinical data)	(Kanbayashi et al., 2010)
Alkylating agents			
Ifosfamide	TRPA1?	Higher neurotoxicity in females	(Schmidt, Baumann, Hanschmann, Geissler, & Preiss, 2001)
Antibody-Drug Conjugates (ADC)			
Brentuximab-vedotin Enfortumab-vedotin	Not investigated	Not disclosed, but likely similar to vincristine as auristatin E as both share a similar inhibitory mechanism	
Bevacizumab	Not investigated	More abdominal pain in females than males with advanced-stage non-small cell lung cancer (clinical trials)	(Brahmer et al., 2011)
Hormonal therapies: Aromatase inhibitors			
Anastrozole	TRPA1	Not studied in male (breast cancer)	
Immunomodulatory drugs			
Thalidomide	TRPA1 TRPV4	Not investigated	
Antimetabolites			
5-Fluorouracil (Capecitabine)	Not investigated	Female patients with advanced colorectal cancer had significantly higher risk for hand-foot syndrome (clinical trials)	(Lévy et al., 1998)
Epothilones			
Ixabepilone	Not investigated	Not studied in male, only in female	
Combination therapies			
leucovorin + fluorouracil + oxaliplatin (FOLFOX)	Not investigated	Higher incidence of peripheral neuropathy and hand-foot syndrome in female patients with colorectal cancer (clinical trials) (Wagner et al., 2021)	(Wagner et al., 2021)
Combined (not mentioned)	Not investigated	Increased I-III grade neurological toxicities in female patients (clinical trials database, $N = 23256$ patients)	(Unger et al., 2022)
Combined	Not investigated	Female sex associated with higher neuropathy sum score after treatment of Hogkin's lymphoma (clinical trials)	(Eikeland et al., 2021)
Combined (ECF, ECX, EOF or EOX)	Not investigated	Males showed higher incidence of all-grade peripheral neuropathy in oesophagogastric cancer	(Davidson et al., 2019)
FOLFOX/ bevacizumab	Not investigated	No differences for grade ≥ 3 sensory neuropathy in patients with unresectable advanced or recurrent metastatic colorectal cancer	(Yamada et al., 2020)
Paclitaxel and/or Oxaliplatin	Not disclosed, but likely TRPV1, TRPA1 and TRPM8	Female sex associated with higher neuropathy score (cohort study of 333 patients)	(Mizrahi et al., 2021)

E: Epirubicin; C: Cisplatin; F: Fluorouracil; X: Capecitabine; O: Oxaliplatin. L: Leucovorin.

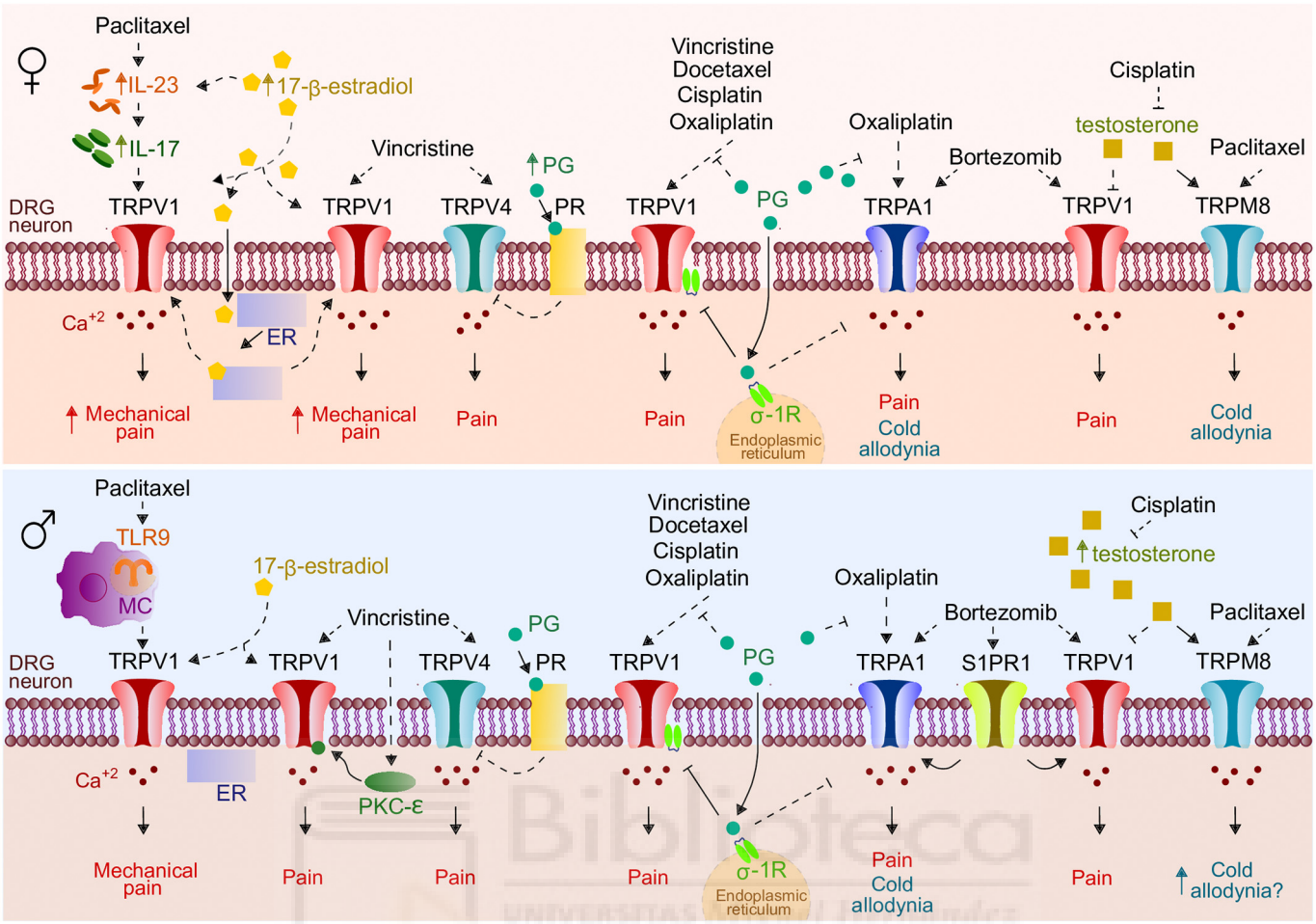

Fig. 3. Schematic representation of potential mechanisms underlying sex dimorphism on CIPN. Paclitaxel-induced mechanical pain was produced through IL-23/IL-17/TRPV1 signaling only in female mice (Luo et al., 2021). The IL-23 induced pain was promoted by the sex hormone 17-β-estradiol. The concentration of this hormone was higher in the peripheral nervous system of females, suggesting a major effect on this sex (Caruso et al., 2013). 17-β-Estradiol also showed a pivotal role on vincristine-induced neuropathy symptoms, since removal of this hormone reduced the greater mechanical hypersensitivity found in female rats compared to male (Joseph & Levine, 2003). As a major mechanism, 17-β-estradiol was found to increase TRPV1 expression through its binding to estrogen receptor (ER) (Payrits et al., 2017). In contrast, progesterone (PG) showed a protective role in the neuropathy induced by cisplatin, docetaxel, vincristine and oxaliplatin (Meyer et al., 2010; Roglio et al., 2009; Zaki et al., 2018). The reduction of pain behavior produced by progesterone could be the result of decreased TRPV1 levels and/or reduced TRPA1 activity through inhibition of σ-1R receptor or, in the case of vincristine, it could also be due to diminished expression of TRPV4 through binding to its progesterone receptor (Jung et al., 2009; Marcotti et al., 2022; Ortíz-Rentería et al., 2018). Plasma levels of progesterone were found to be higher in pre-menopausal women than in men (Tóthová, Ostatníková, Šebeková, Celec, & Hodosy, 2013). As male specific mechanisms, paclitaxel mechanical pain was modulated by TLR9 only in male, possibly through alteration of TRPV1 channel activity (Luo et al., 2019). In addition, inhibition of PKC-ε, a modulator of TRPV1 activity through phosphorylation, only reduced vincristine-induced hyperalgesia in males and ovariectomized females (Joseph & Levine, 2003). Furthermore, higher TRPM8 responses after paclitaxel exposure were observed on male DRG neuronal cultures (Villalba-Riquelme et al., 2022). TRPM8 has also been described as a testosterone receptor (Asuthkar et al., 2015). Testosterone was also postulated to decrease pain by reducing TRPV1 expression (Bai et al., 2018). Levels of this hormone were higher in the peripheral nervous system of males (Caruso et al., 2013). For bortezomib treatment, S1PR1 antagonists only prevented neuropathic pain on male rats (Stockstill et al., 2020). There, S1PR1 could evoke pain through TRPA1 and TRPV1 channels (Kittaka, DeBrecht, & Mishra, 2020). Dashed lines indicating indirect interaction. ER: estrogen receptor. MC: macrophage. PG: progesterone. PR: progesterone receptor. S1PR1: sphingosine-1-phosphate receptor.

role for 17-β-estradiol in CIPN symptoms. Miyamoto et al. (2021) described that estrogen depletion enhanced paclitaxel CIPN in female mice. Selective agonists of the estrogen receptor β (ERβ) reduced the allodynia induced by paclitaxel, oxaliplatin and vincristine (Ma, McFarland, Olsson, & Burstein, 2016). Nonetheless, their efficacy and potency were higher in male rats than in females (Ma et al., 2016). Despite these observations, estrogen antagonists are generally considered for providing pain relief (Paller, Campbell, Edwards, & Dobs, 2009). Indeed, 17-β-estradiol was previously described as a modulator of TRPV1 channel (Lu, Chen, Wang, & Wu, 2009). Due to the higher concentration of this hormone found in the female sciatic nerve, an estrogen-mediated pronociceptive pathway could predominate in this sex (Caruso et al., 2013).

Hormones such as progesterone have been postulated to play a protective role on CIPN development (Falvo, Diviccaro, Melcangi, & Giatti, 2020). In a recent study, a higher incidence of CIPN was found in post-menopausal compared to pre-menopausal women treated with paclitaxel Singh et al. (2022), which could be due to the drop in progesterone levels occurring in menopause. The analgesic effects of progesterone have been evidenced under different chemotherapeutic treatments. Zaki, Mohamed, Motawie, and Abdel Fattah (2018), reported that progesterone ameliorated cisplatin-induced peripheral neurotoxicity. A protective role of this sex hormone was also observed in docetaxel-induced neuropathy (Roglio et al., 2009). In addition, progesterone prevented the neuropathy and exerted an antinociceptive action in docetaxel- and vincristine-treated rats (Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2010; Roglio et al., 2009). Anti-nociceptive effects were also produced by progesterone derivatives such as allopregnanolone, which suppressed oxaliplatin-induced neuropathy (Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2011). A suitable explanation for this protective effect has been previously attributed to progesterone-driven downregulation of TRPV1, TRPA1 and TRPV4 channels (Jung et al., 2009; Ortíz-Rentería et al., 2018). In support of

this tenet, previous studies demonstrated that progesterone decreased TRPV1 plasma membrane levels through inhibition of σ1R receptor, a chaperone that binds to this channel (Ortíz-Rentería et al., 2018). In addition, Marcotti et al. (2022) showed that σ1R modulation of TRPA1 prevented oxalipatin-induced neuropathy. For vincristine-induced CIPN, the protective effect of progesterone could also arise from transcriptional TRPV4 repression through the progesterone receptor (Jung et al., 2009). Hence, progesterone could exert its antinociceptive effect through indirect disruption of TRPV1/TRPV4 channels.

Similarly, testosterone-related modulation of thermoTRP channels has also been shown in events related with cancer treatment. Testosterone has been described as a direct TRPM8 agonist and this channel is highly expressed in prostate cancer (Asuthkar et al., 2015). Furthermore, testosterone was reported to reduce TRPV1 expression in an inflammatory pain model (Bai, Zhang, & Zhou, 2018). Due to these findings and the higher levels of testosterone found in males when compared to female individuals, testosterone has been proposed as a relevant determinant in the sex dimorphism of chronic pain (Roglio et al., 2007; Tanzer & Jones, 2004). However, we have not identified studies investigating the role of testosterone in CIPN. Nonetheless, it was reported that CIPN-inducing agents such as cisplatin and docetaxel inhibited testosterone synthesis and reduced testosterone levels (García et al., 2012; Ryan et al., 2020). Notably, testosterone effect could predominate in males since DRG levels are higher in males than in females (Caruso et al., 2013).

Toll-like receptors (TLR) are immune-related receptors that signal through thermoTRP channels in nociceptors. Notably, sex differences in TLR9 and TLR4 signaling have been described (Luo et al., 2019). Thus, paclitaxel-induced mechanical pain was produced by promoting TRPV1 activity and attenuated by a TLR9 antagonist in male mice. Noteworthy, TLR4 showed a sex-dimorphic effect driving neuropathic pain that was also testosterone dependent (Sorge et al., 2011). TLR4 has also been linked to development of paclitaxel-induced CIPN through modulation of TRPV1 ion channel (Li et al., 2015), and cisplatin and carboplatin were described to act as ligands of TLR4 (Park, Stokes, Corr, & Yaksh, 2014).

Collectively, there are sufficient grounds for a sex dimorphism in CIPN that must be further studied at both clinical and preclinical levels. Clinically, it would be desirable to know the incidence and/or prevalence of the disorder in the different types of cancer and chemotherapeutic treatments. Pre-clinically, it would be important to use both live animal and *in vitro* models to investigate CIPN and its resolution in male and females, along with the effect of sex hormones on its intensity and/or resolution.

3.1. TRP channels in the pathophysiology of CIPN

CIPN can be produced by a wide variety of chemotherapeutic agents such as taxanes (e.g. paclitaxel, docetaxel), vinca-alkaloids (e.g. vincristine), platinum-based compounds (e.g. oxaliplatin, carboplatin, cisplatin), alkylating agents (e.g. ifosfamide), antimetabolites (e.g. 5-fluorouracil), epothilones (e.g. ixabepilone), immunomodulatory drugs (e.g. thalidomide), proteasome inhibitors (e.g. bortezomib) and immunotoxins (e.g. enfortumab) (Saif et al., 2001; Zajączkowska et al., 2019). Capecitabine (5-fluorouracil) provokes a palmar-plantar erithrodysesthesia (referred to as hand-foot syndrome), and even immunotherapy induces skin sensory abnormalities such as itch (e.g. pembrolizumab). These chemotherapeutic agents affect peripheral nociceptor endings to induce painful and disturbing sensory symptoms. Most of these agents alter the activity of the sensory neurons through a direct or indirect potentiation of their excitability. Although each class of compounds acts on different cellular targets inducing oxidative stress, mitochondrial and DNA damage, immunological processes and neuroinflammation, all commonly lead to sensitization of peripheral sensory terminals (Areti, Yerra, Naidu, & Kumar, 2014; Aromolaran & Goldstein, 2017; Lees et al., 2017). A core molecular and cellular mechanism in CIPN appears to be potentiation of nociceptor ion channels involved in the generation and propagation of action potentials (Aromolaran & Goldstein, 2017). Among these nociceptor ion channels, thermoTRP channels that mediate the generation of action potentials in peripheral terminals have emerged as pivotal contributors to the peripheral neuropathy caused by most chemotherapeutic agents (Table 1), particularly of TRPV1, TRPA1, TRPM8 and TRPV4. We turn next to describe the role of these channels in CIPN.

TRPV1 protein has been shown to be upregulated in nociceptors by chemotherapeutic drugs such as paclitaxel, docetaxel, oxaliplatin, cisplatin, bortezomib and vincristine (Table 1) (Chiba et al., 2017; Ertilav, Nazıroğlu, Ataizi, & Yıldızhan, 2021; Quartu et al., 2014; Ta et al., 2010; Villalba-Riquelme et al., 2022). TRPV1-enhanced expression was accompanied by an increase in channel function that led to an increase in neuronal excitability, which underlies the thermal hyperalgesia and mechanical allodynia produced by these agents. Noteworthy, Villalba-Riquelme et al. (2022), using a long-term primary nociceptor culture, reported that paclitaxel increases the expression and channel activity of TRPV1 in peptidergic (IB4(-)) and non-peptidergic (IB4(+)) sensory neurons. This enhanced activity contributed to the higher electrical activity displayed by these nociceptors upon drug exposure. Notably, this sensitizing effect was reversible peaking 48h after paclitaxel exposure and virtually resolving at 96h. Thus, cumulative evidence hints to a key role of this thermoTRP channel in the manifestation of CIPN painful symptoms.

Akin to TRPV1, TRPA1 expression and function was also increased after oxaliplatin, cisplatin, carboplatin, thalidomide and bortezomib treatment (De Logu et al., 2020; Miyano et al., 2019; Nativi et al., 2013; Ta et al., 2010; Trevisan et al., 2013). Aromatase inhibitors such as anastrozole, used as adjuvant therapy for hormone-dependent breast cancer, have been reported to ionotropically activate the TRPA1 channel (Fusi et al., 2014). A possible contribution of TRPA1 to paclitaxel CIPN has also been observed in animal models of peripheral neuropathy (Materazzi et al., 2012; Pittman, Gracias, Vasko, & Fehrenbacher, 2014). However, a recent study that evaluated the direct effect of the taxane in a long-term primary nociceptor culture did not find a significant change in TRPA1 expression or function (Villalba-Riquelme et al., 2022). A role of TRPA1 in ifosfamide-evoked visceral pain has also been suggested, as symptoms were attenuated by the channel antagonist HC-030031 (Pereira et al., 2013). However, no studies were found regarding the role of TRPA1 on ifosfamide-induced peripheral somatic pain in males and females.

Oxaliplatin, bortezomib and thalidomide-induced TRPA1 upregulation may contribute to the mechanical hyperalgesia characteristic of CIPN (De Logu et al., 2020; Li, Deng, Shang, Wang, & Xiao, 2018; Liu et al., 2019). Because TRPA1 has been proposed to be a sensor for noxious cold (<18°C), it has been suggested that this channel may also mediate the cold allodynia that suffer patients treated with oxaliplatin (Zhao et al., 2012). However, the involvement of TRPA1 in cold sensation is under intensive debate with studies supporting and questioning this role (Buijs & McNaughton, 2020). Alternatively, the cold hypersensitivity described after paclitaxel and oxaliplatin treatments was assigned to a potentiation of the TRPM8 channel, a thermoTRP channel gated by cold temperatures (<30°C) and refreshing substances such as menthol (Kawashiri et al., 2012; Villalba-Riquelme et al., 2022). In support of this tenet, TRPM8 mRNA levels increased in an animal model of cisplatin-induced CIPN (Ta et al. (2010)), as well as in long-term primary nociceptor cultures treated with paclitaxel (Villalba-Riquelme et al., 2022). Nonetheless, these studies need to be replicated for a solid support of TRPM8 as the mediator of cold allodynia as several ion channels contribute to this thermal sensation in nociceptors (Buijs & McNaughton, 2020).

Another thermoTRP channel that may be involved in the etiology of CIPN, particularly in mechanical hyperalgesia, is TRPV4, since it is an osmosensitive channel. Indeed, paclitaxel, thalidomide, and vincristine have been reported to increase its functionality (Alessandri-Haber,

Dina, Joseph, Reichling, & Levine, 2008; De Logu et al., 2020; Sánchez, Muñoz, & Ehrlich, 2020). Accordingly, TRPV4 contributed to the mechanical allodynia evoked by these compounds (Alessandri-Haber et al., 2008; De Logu et al., 2020). Less evidence has been found on the role of other thermoTRP channels in CIPN. In this regard, TRPV2 was upregulated in rat DRG treated with cisplatin (Hori, Ozaki, Suzuki, & Sugiura, 2010), although the possible role of TRPV2 on CIPN pain symptoms remains to be elucidated.

Several studies are evaluating thermoTRP channel modulators as a promising strategy for attenuating CIPN symptoms (Singh, Adhya, & Sharma, 2021). Among TRP receptors, TRPV1 is emerging as a key therapeutic target for this type of peripheral neuropathy. Duloxetine, the only medicine for painful CIPN treatment recommended by ASCO society, has shown some efficacy reducing paclitaxel-induced TRPV1 upregulation in rats (Wang et al., 2022), suggesting a mechanism of action for the drug. Furthermore, promising results have been obtained in clinical trials using capsaicin patches that desensitize TRPV1 (Qutenza®) (Privitera & Anand, 2021). As a result, capsaicin reduced pain intensity and improved the quality of life of the patients (Maihofner & Heskamp, 2013). Other clinical trials have also shown relief of CIPN symptoms with this treatment (Anand et al., 2019; Filipczak-Bryniarska et al., 2017), suggesting a high therapeutic potential (Maihöfner, Diel, Tesch, Quandel, & Baron, 2021).

An interesting therapeutic approach for topically targeting TRPV1 in peripheral terminals is the use of soft receptor antagonists (Serafini et al., 2018). TRPV1 soft antagonists are based in the capsaicin scaffold and have shown inhibitory efficacy on TRPV1 receptors at micromolar concentrations, both under basal and inflammatory conditions (Nikolaeva-Koleva et al., 2021). These soft drugs have the unique property of being hydrolyzed by dermal esterases, preventing by this way a systemic distribution that might interfere with the chemotherapeutic treatment. Their systemic administration is short-lived and does not produce hyperthermia (Serafini et al., 2018). Consequently, TRPV1 soft antagonists exhibit a high pharmacological safety. Notably, their local application significantly reduced histamine-induced itch (Nikolaeva-Koleva et al., 2021), paving the way for the development of topical ointments that help to mitigate the sensory symptoms of CIPN. In this regard, a preliminary report of a clinical proof-of-concept study that we performed revealed that an anhydrous topical formulation of the TRPV1 soft antagonist, AG1549 (IUPAC: *2-((4-hydroxy-2-iodo-5-methoxybenzyl)amino)-2-oxoethyl nonanoate)*, significantly alleviated grade I/II paclitaxel-induced CIPN sensory symptoms (IASP World Congress on Pain 2022, poster n° PFR325: *A topical formulation of a soft TRPV1 antagonist (AG1549) alleviates chemotherapy-induced peripheral neuropathy symptoms)*.

In addition, complementary studies are evaluating modulators of other thermoTRP channels as a promising strategy for CIPN symptoms. Application of a topical cream formulated with the TRPM8 activator menthol reduced pain scores in neuropathic pain patients with common CIPN symptoms (Fallon et al., 2015). Furthermore, riluzole, an inhibitor of TRPM8 overexpression, is currently being tested in clinical trials against oxaliplatin CIPN symptoms (Kerckhove et al., 2019), but no data are still available. Collectively, all this information suggests that modulation of thermoTRP channels could be a promising pharmacological strategy for reducing CIPN pain symptoms in male and female patients and this modulation could be adjusted depending on the sex dimorphism promoted by the chemotherapeutic agent or the oncological condition.

4. Outlook

Sex dimorphism in human pathology, and particularly in chronic pain, is an emerging topic that must be considered for designing and developing better treatments that help to reduce the negative impact of this disease in our society. It is becoming evident the existence of a sex hormone – thermoTRP axis that contributes to define differences in chronic pain incidence between women and men. Furthermore, as sex hormones vary throughout our life this proposed axis may also contribute to define the evolution of pain symptoms at different ages, particularly the higher incidence of most of the chronic pain conditions in aged individuals. Although still in its infancy, this axis warrants intense investigation as both sex hormones and thermoTRPs are found in the peripheral and the central nervous system. Notably, some thermoTRP channels are hormone receptors that may contribute to the sexual dimorphism observed in chronic pain. Underpinning the molecular and cellular details of this relationship may provide novel therapeutic targets for sex-specific pharmacological interventions. Apart from validating thermoTRPs as interesting therapeutic targets for analgesic intervention, novel exploitable drug target sites can also be suggested. For instance, the testosterone-binding site in TRPM8 appears as an interesting site for testosterone derivates that specifically target the thermoTRP without acting on the androgen receptor, which could be interesting for treating women suffering of chronic migraine pain (Alarcón-Alarcón et al., 2022). Similarly, it could be a valid approach for improving paclitaxel-induced CIPN treatments in men (Villalba-Riquelme et al., 2022). Because chronic pain can exhibit a strong peripheral component contributed by thermoTRPs, photopharmacology could also become an excellent therapeutic strategy in the near future, along with topical ointment formulations of thermoTRP modulators.

Collectively, the lesson learnt is that preclinical studies must use both male and female-based models to identify similarities and differences in the pathophysiological mechanisms that help to define more personalized therapeutic strategies for patients. In addition, clinical studies should also be sensitive to sex, as a differential driver of pharmacological efficacy, potency and/or side effects of disease treatments. Understanding the underlying mechanisms involved in the sex dimorphism of chronic pain will help to reduce the notable societal burden and cost of this disabling syndrome.

Data availability

Data will be made available on request.

Declaration of Competing Interest

Asia Fernández-Carvajal and Antonio Ferrer-Montiel are inventors of the patent TRPV1 modulator compounds (EP3621950B1) protecting a family of TRPV1 soft antagonists based on the capsaicin scaffold. David Cabañero, Eva Villalba-Riquelme and Gregorio Fernández-Ballester declare no conflict of interest.

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Otra de las cosas geniales de hacer una tesis, es que te permite conocer y compartir experiencias con gente de otros lugares con el mismo interés por la ciencia, tanto en colaboraciones, como en congresos.

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Para construir cualquier cosa, sea una tesis o una casa, se necesitan buenos pilares sobre los que apoyarse. Ya lo decía Descartes, no podemos empezar a construir sobre algo que no tiene unos cimientos sólidos. Y eso es lo que habéis sido vosotros. A mis profesores del IES Jorge Juan, por haber puesto esos primeros pilares en mi educación. Porque esa etapa acabó hace años, pero vuestras enseñanzas me han acompañado y crecido conmigo desde entonces. A Rosa Mones, Juan Damián, don Víctor Palacios, José Ramón Bravo, Loli, José Manuel, Joaquín Abegón, Paco Jordá, José Orozco, Mª

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"…pues muy bien, otro día hablamos otro ratico y te cuento más cosas. Hasta otro día"

Hasta otro día. Te quiero Abuelo