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TARGETING PROKINETICIN SYSTEM TO COUNTERACT EXPERIMENTAL CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY.

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1. Riassunto

La neuropatia periferica indotta da chemioterapici (CIPN) rappresenta uno degli effetti collaterali dose-limitante di numerosi agenti antineoplastici comunemente utilizzati in clinica. Il trattamento della CIPN, con i comuni farmaci analgesici è spesso insoddisfacente, pertanto è necessario lo sviluppo di nuovi approcci terapeutici. La patofisiologia della CIPN è molto complessa e non è stata ancora completamente chiarita; è caratterizzata da meccanismi multipatogenici tra cui: danno mitocondriale, stress ossidativo, alterazioni di canali ionici e neuroinfiammazione (Boyette-Davis et al., 2015; Montague e Malcangio 2017). In particolare, è stato dimostrato che le citochine e chemochine pro- ed anti-infiammatorie prodotte da cellule immunitarie, nonché da glia e microglia a livello dei nervi, dei gangli della radice dorsale (DRG) e del midollo spinale sono coinvolte nell'insorgenza e nel mantenimento del dolore neuropatico (Sacerdote et al., 2013). Tra le chemochine, la famiglia delle prochineticine, ha un ruolo importante nell'immunomodulazione, nell'infiammazione, nella nocicezione, nel dolore infiammatorio e in diversi modelli sperimentali di dolore neuropatico come quello indotto da lesione del nervo sciatico (Lattanzi et al., 2015; Guida et al., 2015) o da diabete (Castelli et al., 2016). Sia nell'uomo che nei roditori, la famiglia delle Prochineticine è composta da due proteine, la Prochineticina-1 (PK1) e la Prochineticina-2 (PK2) (Negri et al., 2007), che legano due recettori accoppiati a proteine G, PK-R1 e PK-R2 (Masuda et al., 2002). Le prochineticine ed i loro recettori sono ampiamente distribuiti nel cervello, nel midollo spinale, nei gangli della radice dorsale, nei granulociti, nei macrofagi e nei linfociti. È stato dimostrato che la PK2 è in grado di indurre un profilo pro-infiammatorio nei macrofagi (Martucci et al., 2006), che a sua volta può portare ad una riduzione delle soglie nocicettive in risposta sia a stimoli termici che meccanici (Negri et al., 2006; Giannini et al., 2009; Maftei et al., 2014). E' stata dimostrata una co-localizzazione dei recettori PK-Rs e TRPV in alcune regioni nervose associate al dolore, ed è stato inoltre evidenziato che se stimolati con la PK2 (*in vitro*) molti neuroni che esprimono i recettori PK sono in grado di rilasciare mediatori del dolore come CGRP e sostanza P (Vellani et al., 2006).

Sulla base di queste considerazioni, il presente progetto ha lo scopo di studiare il ruolo del sistema delle prochineticine in un modello murino di CIPN indotto dalla somministrazione di due diversi agenti chemioterapici comunemente usati in oncologia e correlati allo sviluppo di CIPN: vincristina (VCR), il farmaco più neurotossico tra gli alcaloidi della Vinca e bortezomib (BTZ), il primo inibitore del proteasoma 26s. Questi farmaci, nonostante abbiano meccanismi d'azione diversi sulle cellule tumorali, inducono neurotossicità e conseguente neuropatia attraverso meccanismi molto simili (Flatters et al., 2019; Boyette-Davis et al., 2015).

Tutti gli esperimenti sono stati condotti utilizzando topi maschi del ceppo C57BL/6J di 9 settimane di età. Sulla base dei protocolli più comunemente utilizzati in letteratura, la CIPN è stata indotta negli animali attraverso la somministrazione intraperitoneale (i.p.) di bortezomib 0,4 mg/kg, 3 volte a settimana per 4 settimane consecutive (Boehmerle et al., 2014); mentre la vincristina è stata somministrata quotidianamente per 14 giorni consecutivi alla dose di 0,1 mg/kg (i.p.) (Kiguchi et al., 2008). Gli animali controllo sono stati trattati con il veicolo.

Poichè l'allodinia è uno dei sintomi più comunemente riscontrati sia nei pazienti che nei modelli sperimentali di CIPN, l'allodinia meccanica e termica sono state monitorate durante gli esperimenti, utilizzando rispettivamente il Von Frey test e il test dell'acetone. Inoltre, è stata monitorata l'iperalgesia termica mediante il plantar test. La somministrazione di bortezomib o di vincristina ha indotto negli animali una condizione neuropatica dolorosa caratterizzata dalla presenza di allodinia meccanica, termica e di iperalgesia termica. Per valutare il possibile effetto terapeutico di PC1, un antagonista non peptidico dei PK-Rs, gli animali trattati con BTZ o VCR, in un chiaro stato di ipersensibilità, sono stati sottoposti al trattamento cronico con PC1 (150µg/kg, iniettato per via sottocutanea, due volte al giorno per 14 (BTZ) o 7 (VCR) giorni consecutivi). Indipendentemente dall'agente chemioterapico utilizzato per indurre la neuropatia, il trattamento cronico con PC1 è stato in grado di migliorare significativamente la sintomatologia dolorosa.

Per valutare nel nostro modello (BTZ e VCR), il ruolo del sistema PK, le analisi biochimiche sono state eseguite a due diversi tempi sperimentali: prima dell'inizio del trattamento cronico con PC1 (rispettivamente 14 o 7 giorni dalla prima somministrazione di bortezomib e vincristina) e al termine del protocollo sperimentale (28 o 14 giorni dalla prima somministrazione con BTZ e VCR). Quattordici giorni dopo la prima somministrazione di BTZ, non sono state osservate alterazioni dell'espressione del sistema PK nelle principali stazioni coinvolte nella trasmissione del dolore (nervo sciatico, DRG e midollo spinale). Gli animali trattati con VCR invece, 7 giorni dopo la prima somministrazione di chemioterapico, mostravano, sia a livello del sistema nervoso periferico (DRG) che del sistema nervoso centrale (midollo spinale), un'up-regolazione di entrambi recettori del sistema PK.

È noto dalla letteratura che il reclutamento e l'infiltrazione dei macrofagi a livello dei DRG svolge un ruolo chiave nello sviluppo e nel mantenimento della CIPN (Lees et al., 2017). Inoltre, è stato dimostrato che la PK2 è in grado di indurre i macrofagi a migrare e acquisire un fenotipo pro-infiammatorio, abbiamo quindi deciso di valutare l'espressione dei markers del macrofago/microglia (CD68 e CD11b) e del TLR4, la cui attivazione è correlata alla modulazione delle citochine pro-infiammatorie, nei tessuti nervosi dei nostri animali. A metà del protocollo chemioterapico (14 giorni BTZ e 7 giorni VCR), la sintomatologia dolorosa era correlata ad un aumento dell'espressione di CD68 e del TLR4. Inoltre, negli animali BTZ, le analisi di immunofluorescenza hanno confermato la presenza di macrofagi attivati sia a nel DRG che nel nervo sciatico. Allo stesso tempo (14 giorni BTZ e 7 giorni VCR), nei tessuti nervosi periferici di tutti gli animali sottoposti a trattamento con i chemioterapici, abbiamo osservato un profilo pro-infiammatorio caratterizzato da alti livelli delle citochine pro-infiammatorie IL-1β e IL-6 e bassi livelli dell' anti-infiammatoria IL-10. A questi tempi sperimentali, il sistema nervoso centrale non sembrava essere alterato dalla neurotossicità della chemioterapia.

Al termine del protocollo sperimentale (BTZ giorno 28 e VCR giorno 14), nel nervo sciatico e nei DRG degli animali BTZ, è stato rilevato un profilo neuroinfiammatorio più marcato; l'aumentata produzione di citochine potrebbe essere stata sostenuta dall'infiltrazione di macrofagi attivati. Infatti, negli animali con CIPN, a livello del nervo sciatico e dei DRG abbiamo osservato un up-regolazione del CD68, del CD11b e del TLR4 oltre che un aumento delle cellule CD68 positive nei topi BTZ. Queste cellule esprimevano anche PK2, suggerendo che i macrofagi infiltranti potrebbero rappresentare una fonte di PK2 nei tessuti nervosi periferici. Allo stesso tempo (BTZ giorno 28 e VCR giorno 14), anche il midollo spinale è stato influenzato dalla neurotossicità correlata alla chemioterapia; era infatti evidente un'upregolazione di tutti i componenti del sistema PK, del CD68 e del TLR4 oltre ad un chiaro profilo pro-infiammatorio. Inoltre, è stata osservata una colocalizzazione tra PK2 e GFAP (marker astrocitario) a livello del midollo spinale degli animali BTZ, suggerendo che, in questa stazione nervosa, gli astrociti potrebbero essere tra i principali produttori di PK2. Anche negli animali VCR, a livello del sistema nervoso centrale, era presente un aumento dell'espressione del GFAP e del CD68.

PC1 è stato in grado di contrastare l'attivazione immunitaria e l'amplificazione neuroinfiammatoria, il cui ruolo nel mantenimento dell'ipersensibilità è noto. Infatti, il trattamento con l'antagonista ha portato ad una riduzione dell'espressione del CD68, del CD11b e del TLR4 e ha ristabilito un corretto equilibrio delle citochine sia a livello del sistema nervoso periferico che centrale.

Poiché nella pratica clinica molti pazienti sono sottoposti a multipli cicli di chemioterapia (Starobova & Vetter, 2017), gli animali precedentemente trattati con gli agenti chemioterapici sono stati sottoposti ad un secondo ciclo BTZ o VCR. Dopo l'interruzione del primo ciclo di chemioterapia, gli animali hanno progressivamente recuperato le loro soglie di risposta basali. In entrambi i protocolli (BTZ e VCR), il secondo ciclo di chemioterapia ha indotto nuovamente lo sviluppo di ipersensibilità negli animali precedentemente trattati con lo stesso agente chemioterapico. L'effetto allodinico indotto dai sia da BTZ che da VCR era meno evidente nei topi che erano stati precedentemente trattati anche con PC1 rispetto a quello osservato

negli animali trattati in entrambi i cicli con il solo chemioterapico, indicando un possibile ruolo protettivo di PC1.

Nella seconda parte del nostro studio, considerando la forte suscettibilità del DRG alla tossicità dei chemioterapici (Montague e Malcangio, 2017), abbiamo iniziato a studiare il coinvolgimento dei neuroni nella CIPN usando un modello *in vitro* rappresentato da neuroni sensitivi primari di DRG. In particolare, ci siamo concentrati sulla crescita dei neuriti, un fenotipo morfologico delle cellule neuronali correlato alla loro funzione ed al loro stato di salute (da Silva e Dotti, 2002). I neuroni prelevati da topi wildtype sono stati messi in coltura e sono state testate diverse concentrazioni di BTZ e VCR (Alè et al., 2015). Entrambi gli agenti hanno indotto una riduzione dose-dipendente della lunghezza totale dei neuriti rispetto alle cellule incubate con il solo veicolo. Basandoci sulla letteratura, abbiamo selezionato diverse concentrazioni di PC1 (Severini et al., 2015) che sono state utilizzate in associazione a BTZ e VCR. La dose più efficace di PC1 nel prevenire la tossicità indotta dai chemioterapici si è dimostrata essere la 250 nM.

Abbiamo quindi iniziato ad analizzare i parametri biochimici delle cellule trattate con vincristina da sola o in associazione con PC1. Le cellule VCR hanno mostrato un upregolazione di: PK2, PK-R1, TLR4, IL-1β, IL-6, IL-10 e ATF3; che non era rilevabile nelle cellule co-trattate con VCR e PC1. VCR non ha indotto alterazioni dei livelli di mRNA di PK-R2, GFAP e TNF-α. Quando somministrato in assenza di vincristina, PC1 250nM non ha indotto alterazioni né nella morfologia cellulare né nei livelli di espressione dei parametri sopra citati.

Nel loro insieme i nostri risultati mostrano che il sistema PK è coinvolto nello sviluppo e nel mantenimento della CIPN e, considerando gli effetti positivi del PC1 nel contrastare sia la sintomatologia dolorosa che la neuroinfiammazione, l'antagonismo dei PK-Rs potrebbe rappresentare un nuovo approccio terapeutico per gestire questo tipo di neuropatia dolorosa.

2. Abstract

Chemotherapy induced peripheral neuropathy (CIPN) is a dose-limiting side effect of several antineoplastic agents commonly used in clinics. Up to now its management with common analgesic drugs is often unsatisfactory, therefore novel therapeutic approaches are needed.

The molecular mechanisms underlying CIPN are still largely unknown, even if involvement of mitochondrial toxicity and oxidative stress, ion channels alterations and neuroinflammatory processes have been suggested (Boyette-Davis et al., 2015; Montague and Malcangio 2017). In particular, some cytokines and chemokines are involved in the initiation and persistence of pain (Sacerdote et al., 2013). Among chemokines, the Prokineticin family has an important role in immunomodulation, inflammation, nociception, inflammatory pain and in different experimental neuropathic conditions such as nerve lesion (Lattanzi et al., 2015; Guida et al., 2015) and diabetic neuropathy (Castelli et al., 2016). Both in humans and rodents, Prokineticin-1 (PK1) and Prokineticin-2 (PK2) (Negri et al., 2007) are ligands of two metabotropic receptors known as PK-R1 and PK-R2 (Masuda et al., 2002). Prokineticins and their receptors are expressed in the brain, spinal cord, dorsal root ganglia, granulocytes, macrophages and lymphocytes. It has been demonstrated that PK2 acts on macrophages and induces a pro-inflammatory profile (Martucci et al., 2006), which can lead to reduced nociceptive thresholds to thermal and mechanical stimuli (Negri et al., 2006; Giannini et al., 2009). Furthermore, in some nervous regions associated to pain, a co-localization of PK-Rs and TRPV receptors has been shown, and many neurons that respond to PK2 express and release typical pain mediators such as CGRP and substance P (Vellani et al., 2006).

On the basis of these considerations, our study investigates the role of prokineticin system in a murine model of CIPN induced by the administration of two different chemotherapeutic agents commonly used in oncology and related to CIPN development: Vincristine (VCR), the most neurotoxic drug among Vinca Alkaloids, and bortezomib (BTZ), the first approved 26s proteasome inhibitor. These drugs, despite having different mechanisms of action on tumor cells, induce neurotoxicity and consequent neuropathy with similar mechanisms (Flatters et al., 2019; Boyette-Davis et al., 2015).

C57BL/6J, 9 weeks old, male mice were used in all the experiments. Based on the most common protocols used in literature, CIPN was induced in mice through the intraperitoneal (i.p.) administration of bortezomib 0.4mg/kg, 3 times a week for 4 consecutive weeks (Boehmerle et al., 2014). Vincristine was administered at the dose of 0.1mg/kg, i.p. injected daily for 14 consecutive days (Kiguchi et al., 2008). Control mice were injected with the vehicle.

Allodynia is one of the most frequent symptoms of CIPN, therefore, mechanical and thermal allodynia were monitored using Von Frey and Acetone Drop Test, respectively. Furthermore, thermal hyperalgesia was evaluated by Plantar test. Both bortezomib and vincristine induced in mice a painful neuropathy characterized by the presence of mechanical and thermal allodynia as well as thermal hyperalgesia. To assess the potential therapeutic effect of PC1, a non-peptidic antagonist of PK-Rs, BTZ- and VCR- mice in a clear hypersensitivity condition were subjected to chronic treatment with PC1 (150µg/kg, s.c. injected, daily administered for 14 or 7 consecutive days in the BTZ and VCR protocol respectively). Independently from the chemotherapeutic agent used to induce CIPN, PC1 was effective in ameliorating the painful symptoms.

To assess the role of PK system in our model, for each experimental protocol (BTZ and VCR) we performed biochemical analysis at two different time points: before starting PC1 treatment (BTZ day 14 and VCR day 7), and at the end of chemotherapeutic schedule (BTZ day 28 and VCR day 14). Fourteen days after the first BTZ administration (BTZ day 14), no alterations were detected in PK system expression in the main stations involved in pain transmission (sciatic nerve, DRG, spinal cord). On the other hand, VCR-mice at day 7 (VCR day 7) already showed an upregulation of both PK-Rs at the level of the peripheral (DRG) and central (spinal cord) nervous system.

Recruitment and infiltration of macrophages in DRG is known to have a key role in CIPN (Lees et al., 2017) and since PK2 is able to induce the macrophage to migrate and to acquire a pro-inflammatory phenotype, we evaluated expression of the macrophages/microglia markers (CD68 and CD11b) and of TLR4, whose activation is linked to pro-inflammatory cytokines modulation. At BTZ day 14 as well as VCR day 7, painful symptoms were related to an increase of CD68 and TLR4 expression. Immunofluorescence analysis

confirmed the presence of activated macrophages in DRG and sciatic nerve of BTZ-mice. At the same time points, both BTZ- and VCR- mice peripheral nervous tissues showed a pro-inflammatory profile with high levels of TNF-α, IL-1β and IL-6 and low levels of IL-10. At BTZ day 14 and VCR day 7, the central nervous system was not directly affected by chemotherapy.

At the end of the chemotherapeutic schedule (BTZ day 28 and VCR day 14), a stronger neuroinflammatory profile was detected in sciatic nerves and DRG of mice undergoing chemotherapy. The increased cytokine production could be sustained by infiltrating macrophages since we detected in both nerve and DRG of BTZ- and VCR- treated mice also an upregulation of CD68, CD11b and TLR4 and an increase of CD68 positive cells in BTZ-mice. These cells also expressed PK2, suggesting that infiltrating macrophages could be a source of PK2 in peripheral nervous tissues. At the same time point (BTZ day 28 and VCR day 14), also spinal cord was affected by chemotherapy-related neurotoxicity. In fact, an upregulation of all PK system components, high levels of CD68 and TLR4 and a clear pro-inflammatory cytokine profile were evident. Moreover, a colocalization between PK2 and the astrocytes marker GFAP in the spinal cord of BTZ-mice was observed, suggesting that astrocytes could be the main producers of the PK2 in this nervous station. An upregulation of both GFAP and CD68 was also present in the central nervous system of VCR treated mice.

PC1 was able to counteract immune/neuroinflammatory activation and amplification, which is important to enhance hypersensitivity. In fact, the antagonist prevented or reduced the expression of CD68, CD11b and of TLR4 and restored a correct cytokine balance both in the peripheral and central nervous system.

To more closely resemble the clinical situation, where patients are often subjected to several cycle of chemotherapy (Starobova & Vetter, 2017), mice previously treated with the chemotherapeutic agents, were subjected to a second BTZ- or VCR cycle. Following the interruption of chemotherapy, mice progressively recovered from neuropathy. In both protocols the second chemotherapy cycle induced again hypersensitivity, but the allodynic effect induced by the chemotherapeutic drugs was less evident in mice previously treated with PC1 if compared to that observed in BTZ/VCR-only re-treated mice, indicating a possible protective role of PC1.

In the second part of our study, considering the susceptibility of DRG to chemotherapy (Montague and Malcangio, 2017), we began to study the involvement of neurons in CIPN using an *in vitro* model represented by DRG primary sensory neurons. In particular, we focused on neurite outgrowth, a morphological phenotype of neuronal cells that correlates with their function and health (da Silva et al., 2002). Neurons collected from wildtype mice were cultured and different concentrations of bortezomib and vincristine were tested (Alè et al., 2015). Both agents induced a dose-related reduction of neurite length compared to vehicle-treated cells. Different concentrations of PC1, based on the literature, (Severini et al., 2015) were co-applied to BTZ and VCR. PC1 dose-dependently prevented chemotherapy induced toxicity and the most effective dose of the antagonist was 250nM.

We started to analyse biochemical parameters of neurons exposed to VCR alone or in association to PC1. VCR-cells showed an upregulation of PK2 and PK-R1, TLR4, IL-1β, IL-6, IL-10, and ATF3, which was not detectable in VCR-cells exposed also to PC1. VCR did not induce alterations in PK-R2, GFAP and TNF-α mRNA levels. PC1 250nM alone was tested in the cell cultures: no morphological/biochemical alteration was detected.

Taken together our results show that PK system is involved in the development and maintenance of CIPN and, considering the positive effects of PC1 in contrasting both painful symptomatology and neuroinflammation, PK-Rs antagonism could represent a new therapeutic approach to handle this type of neuropathy.

3. Introduction

3.1 Anatomy and physiology of pain

In the 1979, the *International Association for the Study of Pain (IASP)* defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey, 1979). This definition underlines two important concepts of the phenomenon of pain: its subjectivity and its manifestation also in absence of real tissue damage.

Pain experience is in fact complex and highly variable among individuals. It involves an intricate neurobiological apparatus consisting of dynamic excitatory and inhibitory nervous circuits, deputed to the transmission and elaboration of painful perception. Moreover, the presence of modulatory pathways controlling the emotional state (fear, anxiety, attention and distraction) and the cognitive functions (past experiences, memories of pain) can in turn either enhance or diminish the individual pain experience (Ossipov et al., 2010).

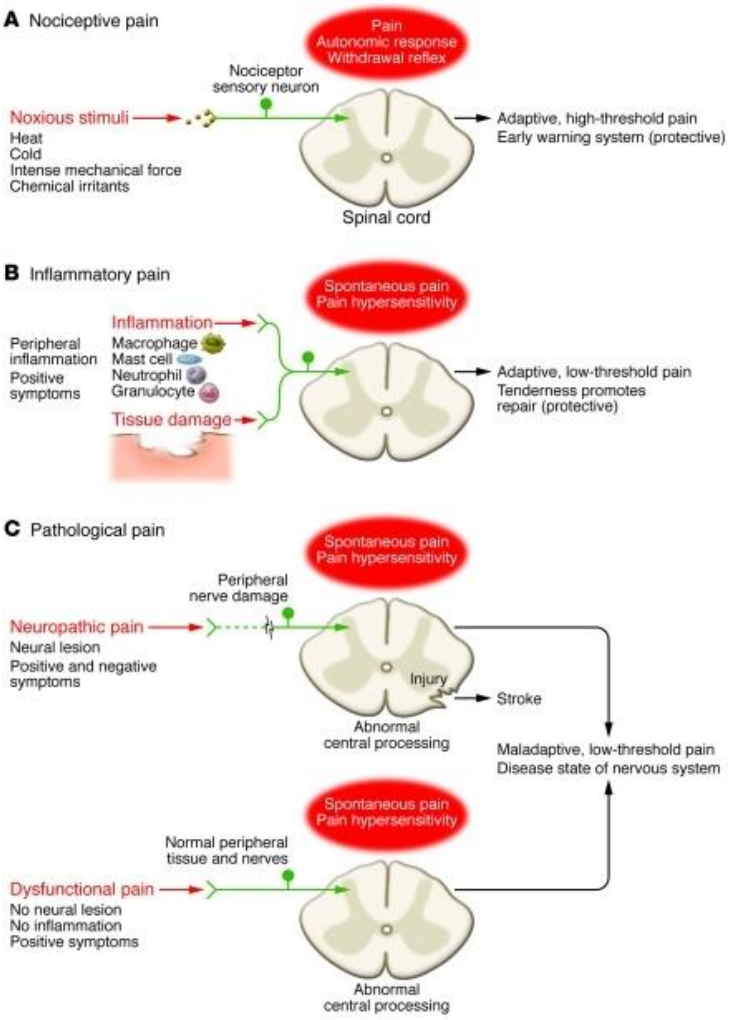


Image 1| Pain classification.
Image from Woolf 2010.

Pain can last for a short (acute) or long (chronic) period, can be caused by several conditions and its processing can imply different neuronal mechanisms. Given the complexity and the heterogeneity of pain manifestation, the distinction of the pathways and molecules implicated in the several types of pain processing is fundamental in order to target the treatments at the mechanisms responsible.

According to Woolf classification, pain can be broadly divided into three main types: nociceptive, inflammatory and pathological pain (Woolf, 2010).

Nociceptive pain is protective and required to maintain body integrity as it represents a primitive protection mechanism evolved as a warning of potential dangerous stimuli and therefore to minimize tissue injury. It follows that the absence of nociceptive pain sensitivity has a negative impact on health; congenital insensitivity to pain due to mutations of genes encoding for channels or molecules involved in pain processing or lacking the ability of pain perception due to a sensory damage for instance, typically results in bone fractures, self-mutilation, maintaining of unrealized infections and a reduction of the life span (Axelrod and Hilz, 2003; Raoulf et al., 2010). Inflammatory pain has also a physiological and protective role. This type of pain takes place in presence of tissue injuries or infections and it is associated to an inflammatory state caused by the activation of the immune system. The consequent development of hypersensitivity has a functional role in fact it protects the injured tissue until healing occurs, by minimizing contact with stimuli potentially able to provoke additional pain.

Usually, either nociceptive or inflammatory pain trigger adaptive responses and they last until the resolution of the initial injury. The switch from a physiological to a pathological condition occurs in presence of damage (neuropathic pain) or an abnormality functioning (dysfunctional pain) of the nervous system. In these cases, pain is not protective but maladaptive, and is maintained for a long period ranging from weeks to years. In other words, pathological pain is not a symptom of some disorders but a disease of the nervous system (Woolf, 2010).

3.1.1 Pain pathways

Nociception is the neuronal process that allows people to experience pain. Four distinct steps occur along the pain pathways:

I. Transduction of noxious (mechanical, thermal or chemical) stimuli in neural electrical activity by the activation of ion channels, including transient receptor potential channel subtypes (TRPA, TRPM and TRPV), sodium channel isoforms (Nav), potassium channels subtypes (KCNK) and acid-sensing ion channels (ASICs), clustered on nociceptors (Grace et al., 2014);

II. Transmission of nerve impulses from the site of transduction to spinal cord, brainstem, thalamus, and central structures in the brain;

III. Modulation of nociceptive signals through endogenous mechanisms of pain control (spinal inhibitory interneurons and descending pain modulatory circuits);

IV. Perception of pain as the final result of nociceptive stimulus travelling through the entire nervous system, including the supra-spinal structures involved in memory, cognition and emotion, which contribute to the neuronal network of conscious experience of pain (Fields and Basbaum, 1999).

Nociceptive ascending pathway begins in periphery with the activation of the nociceptors, sensory neurons able to detect dangerous or potentially damaging stimuli for the organism.
Nociceptors are pseudounipolar neurons with a single axon that branches out peripheral and central processes. The afferent branch projects to skin, mucosa, blood vessels and connective tissue of visceral organs, while the other runs to central nervous system (CNS). Specialized structures, at the terminal end of nociceptors, when are in contact with noxious stimuli, convert painful messages in electrical signals that propagate along this axonal pathway from periphery to the spinal cord or hindbrain. From here the signals reach the supra-spinal structures and, finally the cortical and subcortical regions, where painful information is interpreted and perceived.
Unlike other types of sensory fibers, nociceptors have high activation thresholds; in fact in normal conditions, they can be excited only by stimuli intense enough to cause a real tissue damage, and not by innocuous stimulations such as light touch or vibrations. Extreme temperatures, intense pressure and irritant chemicals are considered high threshold stimuli able to activate nociceptors (Dubin and Patapoutian, 2010).
The main primary afferent fibers that convey noxious stimuli to CNS arise from nociceptive neurons having a small or medium-diameter cellular body located in the dorsal root ganglia (DRG). They comprehend the thinly myelinated, mostly mechanosensitive Aδ fibers and the unmyelinated, polymodal, i.e. able to detect mechanical, thermal and chemical stimuli, C fibers (Julius and Basbaum, 2001).

All Aδ fibers respond to intense mechanical stimulations but according to their responsiveness to noxious heat can be divided in type I and type II fibers. The first type includes capsaicin-insensitive fibers responding to high temperatures (52-56°C), while the latter population comprehends capsaicin-sensitive fibers responsive to noxious heat of 40-45°C (Giordano, 2005).
Unmyelinated C fibers represent the majority of the primary afferents fibers and, according to their ability of synthesizing neuropeptides, mostly substance P (SP) and calcitonin gene related peptide (CGRP), are broadly divided in two main populations: peptidergic and non-peptidergic.
Aδ and C nociceptive fibers differ also for their conduction velocity. When activated, Aδ fibers transmit nociceptive potentials with a speed ranging from 12 to 30 m/s leading to a rapid pain sensation, on the other hand C fibers propagate noxious information more slowly (0.5-2 m/s) inducing a second, delayed response to pain. The different pattern of signal propagation results in two consecutive and qualitatively distinct pain sensations: an initial brief, pricking, and well localized "first" pain and a longer-lasting, burning, and less well localized "second" pain (Ploner et al., 2002).
Another group of nociceptors is represented by C nociceptive fibers known as "silent" or "sleeping" nociceptors which respond to noxious stimuli only when sensitized by tissue injury or inflammation.
Peripheral primary afferents also include myelinated, fast conducting (30-100 m/s) Aβ fibers arising from DRG large-diameter neurons, responsive to innocuous mechanical stimuli. Normally they do not contribute to pain sensation but in some cases they begin to signal in painful response to non-noxious stimuli. Information transmitted by Aβ fibers can be in fact greatly altered during disease conditions or after tissue damage has resolved, leading to abnormal pain signaling (Milligan and Watkins, 2009).
The information collected by nociceptive fibers in periphery reaches the spinal cord via the dorsal roots. Here (spinal cord) their central processes form synapses with different populations of second-order neurons, mostly distributed in the superficial layers of the dorsal horns (Rexed laminae I and II). Nociceptors also synapse in some deeper laminae such as Rexed laminae V and X. Dorsal horns neurons include Nociceptive Specific (NS) neurons, exclusively responsive to nociceptive input conveyed by Aδ and C fibers, Wide Dynamic Range (WDR) neurons that respond to both nociceptive and non-nociceptive stimuli coming from Aδ, C and Aβ central axons and Non-Nociceptive (N-NOC) neurons responding to innocuous stimulations mostly propagated by Aβ fibers (Almeida et al., 2004).
Nociceptive fibers transmit the nociceptive message through the release of glutamate, as the primary neurotransmitter, and others neuropeptides, which have the potential of exciting second-order nociceptive projection neurons (Grace et al., 2014).
The excitatory amino acid binding to postsynaptic glutamate AMPA and kainate receptors is responsible for a fast synaptic transmission and a rapid, short-term depolarization of second-order neurons. On the contrary a slow and long-term synaptic transmission is mediated by substance P and CGRP release.
Spinal projection neurons relay the nociceptive signals received to higher centres of the CNS following the ascending spinal tracts. Secondary afferents decussate and pass up the spinal cord to midbrain via the spinothalamic, spinoreticular and spinomesencephalic tracts to the thalamus and to sensory cortex, but also have other links, such as to reticular formations, limbic and hippocampal areas (Farquhar-Smith, 2008).
Cortical and supra-spinal areas form the "pain matrix", i.e. a collection of brain regions involved in neurological functions, including cognition, emotion, motivation and sensation, as well as pain, which acting together in pain modulation context lead to the conscious experience of pain (Ossipov et al., 2010).
As mentioned above the three main ascending pathways linking the dorsal horns to brain are:

- ***Spinothalamic tract (STT)***

This tract originates from spinal WDR, NS and N-NOC neuron types propagating noxious and innocuous signals that are related to pain, temperature and touch. Before ascending, secondary axons decussate

transversely through the anterior commissure of the spinal cord. During its passage through the brain stem, STT originates collateral branches destined to reticular formation of the medulla, pons and midbrain, periaqueductal grey matter (PAG), hypothalamus and medial and intralaminar thalamic nuclei (Almeida et al., 2004).

- ***Spinoreticular tract (SRT)***

This tract mostly originates from spinal WDR and NS neurons. It presents two components in the brain stem; one is directed at the precerebellar nucleus in the lateral reticular formation involved in motor control, while the other one is directed to the medial pontobulbar reticular formation involved in mechanism of nociception (Millan, 1999). SRT is an important pathway for the modulation of the nociceptive segmental pathway by activating brain stem structures responsible for descending suppression (Almeida et al., 2004).

- ***Spinomesencephalic tract (SMT)***

As SRT, this tract is highly involved in the mechanisms of pain modulation. This area originates from spinal WDR and NS neurons and projects to different regions of PAG. The projections to the midbrain PAG matter from spinal neurons are functionally distinct; those that reach the PAG in the portion more dorsal to the limiting sulcus have excitatory characteristic in afferent nociceptive transmission, while those that project more ventral to the limiting sulcus activate the mechanisms responsible for the inhibition of the afference of this same pathway (Almeida et al., 2004).

3.1.2 Pain modulation

Pain sensory experience goes beyond the activation of nociceptors as it involves an intricate neuronal network consisting of dynamic excitatory and inhibitory nervous circuits which modulate pain experience by enhancing or inhibiting pain sensation.

In the dorsal horn of spinal cord local GABAergic and glycinergic interneurons have a functional role of inhibition of pain information processing. As described by Melzack and Wall in the *Gate Control Theory of Pain* (1965), these inhibitory interneurons function as a gate in spinal cord regulating the transmission of pain message from primary afferents to the spinal second-order projection neurons. Aβ non-nociceptive fibers activity inhibits (or “closes”) the gate inducing the activation of local inhibitory interneurons, while the activity of small nociceptive C and Aδ fibers eases (or “opens”) the gate (Moayedi and Davis, 2013). The balance between inputs from nociceptive and non-nociceptive primary afferents, which is controlled by the complex spinal inhibitory interneuron circuits, determines the status of this gate, i.e. whether and how intensively the nociceptive signal will be transmitted, via secondary projection neurons, to the higher brain centers (Guo and Hu, 2014). Descending fibers originating from supra-spinal regions which project to the spinal dorsal horns are also implicated in the modulation of this gate. (Moayedi and Davis, 2013).

A pain modulation exists also in the form of descending inhibitory pain pathway which involve different regions within CNS, including cortex, thalamus and brainstem.

The midbrain periaqueductal grey region (PAG) and the rostral ventromedial medulla (RVM) are particularly important in the endogenous modulation of pain. These regions are known to be involved in endogenous pain control through PAG-RVM-spinal cord descending inhibitory pain pathway (Fields, 2006).

These supra-spinal sites exert influences on the perception of pain either directly, sending projection neurons to the spinal cord, or indirectly, sending projection neurons to other regions in the brainstem which in turn send projections to the spinal cord. These modulatory effects are predominantly mediated by descending pathways that use serotonin, norepinephrine and endogenous opioids.

Monoamines and endogenous opioids modulate not also the release of neurotransmitters from nociceptive afferents but also the excitability of dorsal horn neurons by binding different receptor subtypes.
Stimulation of PAG and RVM was found to cause release of serotonin in spinal cord (Cui et al., 1999); at this level serotonin has either inhibitory or facilitatory role in pain transmission which is dependent on the activated receptor subtype (Suzuki et al., 2004; Dogrul et al., 2009).
Also norepinephrine strongly contributes in anti-nociceptive mechanisms associated to descending inhibition. In order to modulate pain, PAG and RVM communicate with an important noradrenergic site: the locus coeruleus (LC) which is one of the major source of direct noradrenergic projections to spinal cord involved in the inhibition of presynaptic and post-synaptic spinal pain transmission neurons (Fields et al., 2005; Proudfit, 1992).
Descending pain inhibition from PAG was also observed after direct microinjection of opioids (Fang et al., 1989; Ossipov et al., 2010).
"On-cells" and "off-cells" are two classes of pain modulatory neurons identified in the RVM and PAG (Mason, 1999). "Off-cells" are excited by opioids and inhibit ascending noxious stimuli from the periphery by triggering descending inhibition. On the contrary, "on-cells" are thought to trigger descending facilitation (Mason, 1999; Fields, 2000).

3.1.3 Mechanism involved in the transition from physiological to pathological pain processing

- Peripheral sensitization

Tissue injury and nerve damage produce pain hypersensitivity inducing molecular and cellular changes in the primary afferent neurons. This neuronal plasticity manifests through an increased responsiveness and reduced activation threshold for thermal and mechanical stimuli of nociceptors and is referred as peripheral sensitization.
These electrophysiological dysfunction correlate to behavioral phenomena which include spontaneous pain, hyperalgesia (increased responses to noxious stimuli) and allodynia (nociceptive response to innocuous stimuli) (Cheng and Ji, 2008).
The ability to produce increases in sensitivity after injury is functional and protective in physiological pain; however peripheral sensitization can be a leading cause for the development of persistent pathological pain.
Increases in pain sensitivity are mediated by the local release of inflammatory mediators (collectively referred as "inflammatory soup") from different non-neuronal cells, including fibroblasts, mast cells, neutrophils, monocytes and platelets. After nerve damage, these mediators can also be released by Schwann cells and damaged neurons and primary afferents terminals (Campana, 2007).
"Inflammatory soup" includes prostaglandins E2 (PGE2), bradikinin, adenosine triphosphate (ATP), protons, nerve growth factor (NGF) and pro-inflammatory cytokines (TNF-α and IL-1β) (Julius and Basbaum, 2001).
Furthermore algogen stimuli, such as bradikinin, play an important role in pain processing. Bradikinin is released from kininogen precursors at the site of tissue injury and inflammation and act on its constitutively expressed G-protein-coupled receptors (GPCRs) present on primary sensory neuron peripheral terminals (Prado et al., 2002).
This and others mediators stimulate/activate nociceptors directly (e.g. protons, ATP and 5-hydroxytryptamine (5-HT)) or by increasing sensitivity to subsequent stimuli (e.g. prostaglandins, leukotrienes and NGF) via receptor-mediated second messenger action.
Cyclic AMP, protein kinase A (PKA) and protein kinase C (PKC) are important second messenger and effectors involved in nociceptor sensitization, which may be at least in part caused by changes to ion channels.

Ion channels, including voltage-gated sodium ion channels, acid-sensing ion channels and the heat and capsaicin receptor, transient receptor potential channel V1 (TRPV1), have a significant contribution to activation, sensitization and consequently hyperalgesia and allodynia development, and their sensitivity is strongly regulated by inflammatory mediators (Farquhar-Smith, 2008).
Beyond to activate PKC and PKA pathways, the inflammatory mediators released after injury also activate MAPK signaling in nociceptive primary sensory neurons. Activation of this pathway results in the regulation of transcriptional and translational factors which ultimately lead to increased synthesis of gene encoding for ion channels (such as TRPV1, TRPA1, tetrodotoxin-resistant (TTX-R) sodium and calcium channels), neuromodulators (BDNF, SP and CGRP) and pro-inflammatory cytokines TNF-α and IL-1β. Persistent increase in the synthesis of these pro-nociceptive mediators in primary sensory neurons maintains hypersensitivity of these neurons and persistent pain (Cheng and Ji, 2008).
In addition, nociceptors activation not only results in the transmission of pain message to spinal cord (and from here to brain), but is also responsible for the initiation of neurogenic inflammation.
The antidromic release of peptides and neurotransmitters, notably substance P and CGRP, from activated nociceptors induces in fact vasodilation, plasma extravasation and activation of immune cells, which in turn contribute additional elements to the inflammatory soup (Julius and Basbaum, 2001).

- Central sensitization

Several chemical signals in the spinal cord trigger pain transmission in response to incoming noxious stimuli, including substance P, glutamate, CGRP and many other neuromodulators. Under normal, non-pathological condition, low-frequency activation of Aδ and C fiber nociceptors leads to glutamate release from the central presynaptic afferent nerve terminals in the spinal cord dorsal horn, where the excitatory amino acid induces a short-term activation of glutamate AMPA/kainate receptor subtypes. Although glutamate also binds the NMDA ionotropic glutamate receptor (NMDAR) present on the pre- and post-synaptic neurons, the receptor remains silent, i.e. it does not lead to changes in membrane potential and subsequent pain-projection neuron excitation, because it is plugged by Mg^{2+} (Milligan and Watkins, 2009).
Intense, repeated and sustained activity of primary sensory neurons elicits changes in neuronal and biochemical processing at central synapses and descending projections, transitioning these sites into a pain facilitatory state (Basbaum and Scherrer, 2009; Ossipov et al., 2010). In the spinal dorsal horn, these changes are collectively known as central sensitization and windup.
These processes involve the phosphorylation of a range of receptors, including AMPA and/or kainate receptors, which increases synaptic efficacy by altering channel opening time, increasing burst firing, removing the Mg^{2+}-mediated channel blockade at the NMDA receptor, and promoting trafficking of receptors to the synaptic membrane (Latremoliere and Woolf, 2009).
As a consequence of central sensitization, spinal dorsal horn neurons increase ongoing activity, expand their receptive field and increase their responsiveness by lowering of excitation thresholds (Farquhar-Smith, 2008).
During this time, altered low-threshold non-nociceptive Aβ sensory fibers activate spinal pain-projection neurons, contributing to development of allodynia.
Substance P and CGRP released from primary afferent neurons also contribute to central sensitization by the activation of the NMDA receptor in persistent pain states. Neurokinin A and B acting on neurokinin receptors may influence the NMDA receptors directly by inducing a slow depolarization (by decreased potassium ion conductance).
Nerve growth factor (NGF) induces stimulation of neuropeptide formation and release, which contribute to the development of central sensitization and hyperalgesia. Brain-derived neurotrophic factor (BDNF) is produced by NGF-dependent nociceptors, and its synthesis is increased with inflammation. BDNF augments

spinal neuron excitability by phosphorylation-mediated stimulation of the NMDA receptor (Farquhar-Smith, 2008).
Sustained nociceptor activation caused by peripheral nerve injury and inflammation leads to central sensitization, where enhanced responsiveness of neurons in the spinal dorsal horn is thought to underlie chronic hyperalgesia and allodynia (Chapman et al., 1998; Kidd and Urban, 2001).

3.2 Neuropathic pain

Neuropathic pain is a pathological condition that occurs secondarily to injury of the central and/or peripheral nervous system. Neuropathic pain is an emerging pathology affecting million people in the world for which, nowadays, there are no valid treatments. In fact it is partially unresponsive to classical analgesics and its treatment with adjuvant drugs, i.e. antidepressant or anticonvulsants, provides only a temporary pain relief in a small percentage of patients.
Chronic pain interferes with different aspects of the patient's life, negatively affecting their daily activities, physical and mental health, family and social relationships, and their interactions in the workplace. This problem obviously affects the healthcare system, not only because of health and social care costs but also from the loss of productivity. For these reasons, nowadays, the development of new and efficacious therapies to contrast painful neuropathy represents a priority.
Neuropathic pain arises from lesions to both the central and peripheral nervous system and in the human many etiologies have been recognized, including (Sacerdote et al., 2013):
- mechanical nerve injuries/compression;
- spinal cord injuries;
- metabolic diseases (e.g. diabetes);
- viral diseases (e.g. herpes zoster, HIV);
- inflammatory/immunological mechanism (e.g. Multiple sclerosis);
- alcoholism (vitamin B12 deficiency);
- iatrogenic: chemotherapy of cancer, AIDS or tuberculosis (e.g. cis-platinum);
- vascular lesions of the hypothalamus;
- congenital (e.g. Charcot-Marie-Tooth);
- aging.

Regardless the cause, neuropathic patients typically exhibit a mixture of sensory loss with ongoing spontaneous pain and enhanced sensitivity to innocuous stimuli, i.e. allodynia. Mechanical and thermal hyperalgesia (increased pain response to painful stimuli) are also typical frequent symptoms (Treede et al., 2008; Zimmermann, 2001; Colleoni and Sacerdote, 2010).
The majority of the initial research on the pathophysiological basis of neuropathic pain focused on the plasticity properties of neurons following a nerve injury, leading to the proposal of both peripheral and central sensitisation as important disease mechanisms (Sacerdote et al., 2013).
Peripheral and central amplification is mediated by changes in the expression and distribution of ion channels (sodium and calcium channel); expression of receptors and neurotransmitters; increased neuronal excitability and ectopic generation of action potentials; axonal atrophy, degeneration or regeneration (Wallerian degeneration); damage to small fibres; neuronal cell death and reorganization of central nociceptive circuits (Costigan et al. 2009; Latremoliere and Woolf, 2009). Loss of spinal inhibitory control and changes in the balance of facilitation/inhibition within descending pain modulatory pathways are other mechanisms which contribute to neuropathic pain development (Tesfaye et al., 2013).

Recently it has emerged that neuropathic pain pathogenesis and maintenance also involve a pathological interaction between neurons, inflammatory immune cells and glia cells, as well as a wide cascade of pro- and anti-inflammatory cytokines (Austin and Moalem Taylor, 2010; Calvo et al. 2012). Neuronal injury therefore, not only results in profound modifications of the activity of sensory neurons and their central projection pathways, but is also coupled to a sustained immune-inflammatory response at different anatomical locations associated to chronic pain processing, i.e. nerves, DRG, spinal cord and brain (Calvo et al., 2012).

3.2.1 Neuropathic pain triad: neurons, immune cells and glia

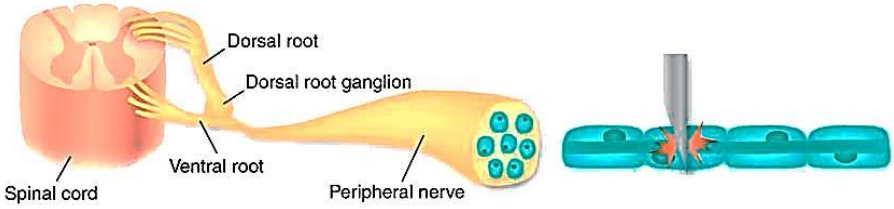


Image 2| Peripheral nerve injury.
Image edited by Scholz & Woolf, 2007.

Peripheral nerve injury causes the activation of resident cells as well as the recruitment of immune cells at the site of injured nerve, in the DRG, and in the spinal cord. Communication among immune cells and immune-like glial cells in the stations of pain transmission is driven by a plethora of immune cell-derived inflammatory cytokines and chemokines, which are crucial mediators for the development and maintenance of persistent pain.

- Neuroimmune interactions in injured nerve

Macrophages act as pivotal mediators in the peripheral inflammatory reactions to nerve lesion. Immediately after nerve injury, resident macrophages rush to the lesion site like a rapid-response team (Mueller et al., 2001).

Neutrophils also participate in the very early immune response to nerve injury, particularly during the first 24 hours after injury, reinforcing macrophage recruitment through the release of chemoattractans and cytokines (Perkins and Tracey, 2000).

Activated macrophages and Schwann cells produce matrix metalloproteases that interrupt blood-nerve barrier (Shubayev et al., 2006). In addition, vasoactive mediators including CGRP, substance P, bradykinin and nitric oxide released from injured axons promote further invasion of monocytes and lymphocytes at site of injury by increasing vascular permeability. As results of these vascular changes, two days after injury, a dense cellular infiltrate predominantly composed by macrophages, T lymphocytes and mast cells, form at the lesion site (Scholz and Woolf, 2007).

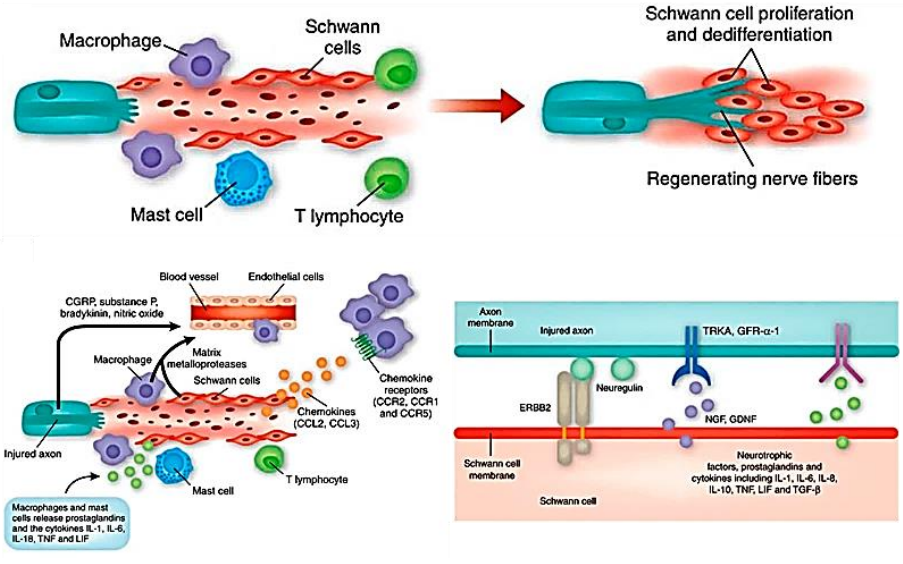


Image 3| Peripheral nerve injury.
Image edited by Scholz & Woolf, 2007.

Resident and infiltrating immune cells as well as Schwann cells release pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 that contribute to axonal damage, and several other factors including chemokines, PGs and NGF, responsible for the initiation and maintenance of sensory abnormalities after injury.
Macrophages and Schwann cells are also implicated in the Wallerian degeneration of axotomized nerve fibres distal to nerve lesion. Macrophages remove by phagocytosis dead or dying remnants of injured Schwann cells, axotomized axons and myelin debris (Bruck, 1997), thus facilitating the reorganization of Schwann cells in order to axonal repair.
Schwann cells, in turn, release chemical signals, such as NGF and GDNF, that promote axonal growth and remyelination (Esper and Loeb, 2004). However, these growth factors induce pain initiation directly activating and sensitizing nociceptors (Malin et al., 2006).
The inflammatory responses to nerve injury driven by resident and infiltrating cells, particularly by macrophages, directly contribute to pain hypersensitivity. Hyperalgesia is delayed in genetically manipulated mice, in which Wallerian degeneration and the recruitment of macrophages in response to nerve injury are delayed (Myers et al., 1996; Araki et al., 2004). Moreover, systemic depletion of macrophages reduces mechanical hypersensitivity after peripheral nerve injury in animal models, revealing their crucial role in the generation of neuropathic pain (Liu et al., 2000).
Uninjured fibers also contribute to the development of pain. Crossing the degenerating environment they change their biologic properties with increased spontaneous activity and up-regulate nociceptive molecules like TRPV1, cytokines and chemokines. They also develop an enhanced responsiveness to pro-inflammatory cytokines.

- Neuroimmune interactions in DRG

Normally, macrophages and few T lymphocytes reside in DRG but their number increase after nerve damage, a condition when also satellite glia cells begin to proliferate.

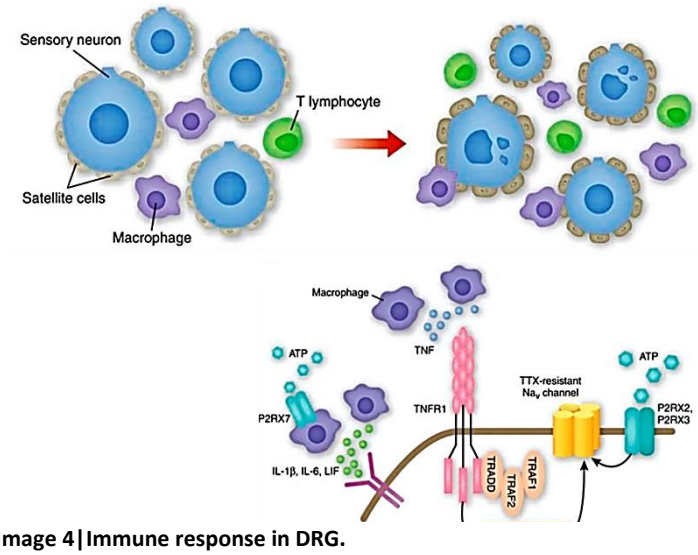


Image 4|Immune response in DRG.
Image edited by Scholz & Woolf, 2007.

These resident immune and glia cells strongly react to nerve injury and their response is reinforced by invading macrophages and T cells. Injury-induced macrophages invasion appears to be triggered by the release of chemokines from DRG neurons (Zhang and DeKoninck, 2006). The accumulation of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines in the DRG after injury contributes to the sensitization of sensory neurons (Levin et al., 2008; Uceyler et al., 2007).
TNF-α acts to increase the density of tetrodotoxin-resistant (TTX-R) sodium channel currents within DRG neurons (Jin and Gereau, 2006), while other cytokines such as IL-6 regulate the synthesis of neuropeptide transmitters. The resultant changes in the sensory neurons phenotype is likely to alter the efficacy of their synaptic input to the spinal cord (Scholz and Woolf, 2007).
Moreover, it was found that deletion of IL-1β receptor or IL-1RA overexpression inhibit the development of spontaneous sensory neuron firing, while blocking IL-1β or IL-6 mediated signalling attenuates neuropathic pain-like behavior (Arruda et al., 2000; Wolf et al., 2006).

- Neuroimmune interactions in spinal cord

Spinal cord glia activation is a common underlying mechanism that leads to development and maintenance of chronic pain.

Microglia and astrocytes have in fact a well-documented role in pain facilitation, modulating neuronal synaptic function and excitability by various mechanisms (Halassa et al., 2007; Pocock and Kettenmann, 2007).

In spinal cord and supra-spinal sites within CNS microglia predominate in the early glial response, subsequently followed by activation and proliferation of astrocytes.

CGRP, substance P, glutamate and ATP released from the presynaptic terminals of the primary afferents after nerve injury determine the activation of spinal microglia and astrocytes.

Once become activated, these cells release immune mediators which diffuse and bind to receptors on presynaptic and postsynaptic terminals in the spinal dorsal horn to modulate excitatory and inhibitory synaptic transmission, resulting in nociceptive hypersensitivity.

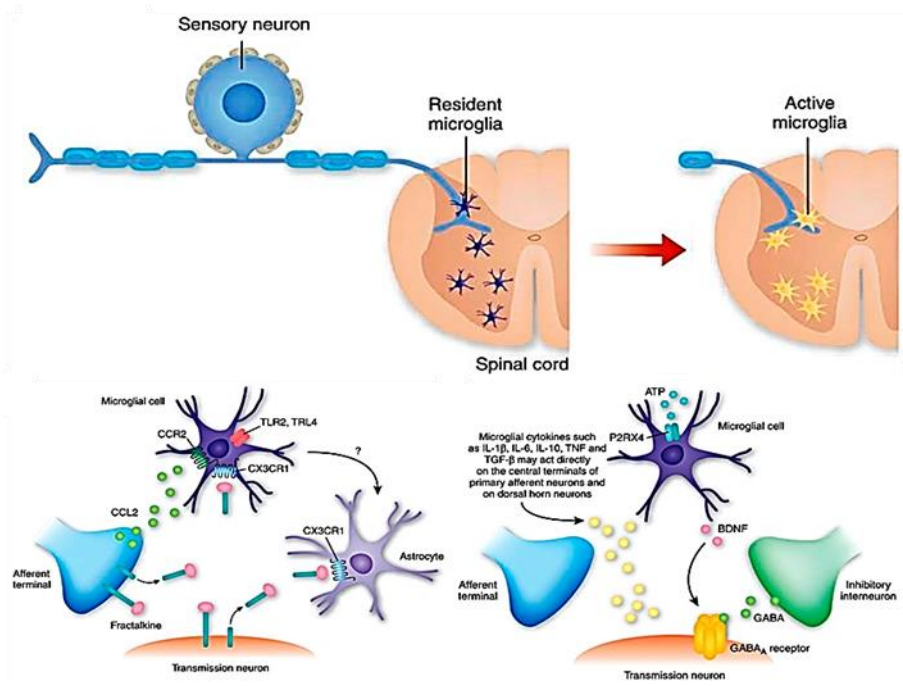


Image 5| Recruitment and activation of spinal microglia and astrocytes.
Image modified from Scholz & Woolf, 2007.

The release of inflammatory mediators, such as TNF-α, IL-1β, IL-6, nitric oxide, ATP and prostaglandins initiates in fact a self-propagating mechanism of enhanced cytokine expression by microglial cells. The production and subsequent release of pro-inflammatory cytokines from activated microglia cells leads to further activation of neighboring astrocytes (Watkins and Maier, 2003).

The activation of astrocytes results in the prolongation of a pain state (Dinarello, 1999). TNF-α, IL-1β and IFN-γ, chemokines and reactive oxygen species (ROS) directly modulate excitatory synaptic transmission at central terminals by enhancing glutamate release. Their effect is partly due to the activation of transient receptor potential channel subtypes (TRPV1 and TRPA1), and the functional coupling between IL-1β receptor and presynaptic glutamate receptors (NMDAR) (Grace et al., 2014).

TNF-α and IL-1β from astrocytes increase neuronal excitability and synaptic strength by increasing the conductivity of glutamate AMPA and NMDA receptors, as well as by increasing the trafficking and surface expression of glutammate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid) receptors, which render neurons vulnerable to excitotoxicity (Stellwagen and Malenka, 2006).

IL-1β also induces the phosphorylation of specific NMDA receptor subunits (i.e. NR1 and NR2A or NR2B subunits) leading to its activation (Zhang et al., 2008; Viviani et al., 2003).

NMDA receptor channel opening leads to the influx of calcium and to increased production of NO and PGE2, which are involved in amplifying the excitability of pain-projection neurons (Milligan and Watkins, 2009).
Excitatory synaptic transmission is further indirectly enhanced following astrogliosis by IL-1β and TNF-α, as the spinal astrocyte glutamate transporters, excitatory amino acid transporter 1 (EAAT1) and EAAT2 are persistently downregulated after peripheral nerve injury, leading to excitotoxicity and nociceptive hypersensitivity (Xin et al., 2009; Ramos et al., 2010).
Cytokines, chemokines and ROS also decrease GABA and glycine release from interneurons and inhibitory descending projections, leading to spinal cord pain circuits disinhibition, phenomenon known to be implicated in the genesis of central sensitization and chronic pain (Latremoliere and Woolf, 2009). Brain-derived neurotrophic factor (BDNF) released as a consequence of microgliosis, also contributes to pain inhibition/ disinhibition by causing a depolarization shift that inverts the polarity of currents activated by the inhibitory neurotransmitter GABA in spinal second-order nociceptive projection neurons (Milligan and Watkins, 2009).
It is also important to highline that either in peripheral nerve and DGR or in CNS a complex network of regulatory circuits controls immune signalling after neuronal insult. These mechanisms include the production of anti-inflammatory mediators and the polarization of specialized immune and glia cells with an anti-inflammatory phenotype to prevent uncontrolled inflammation (Grace et al., 2014)
Alternatively activated microglia (also known as M2 macrophages), sub-populations of T lymphocytes (i.e. Th2 and Treg cells) as well as macrophages contribute to the resolution of nociceptive hypersensitivity after nerve injury, by releasing naturally anti-inflammatory mediators which include IL-10, IL-4 and IL-1RA.
IL-10 and IL-4 cytokines indirectly inhibit the synthesis of pro-inflammatory/pro-nociceptive cytokines and chemotactic factors by microglia, T-cells and macrophages regulating and promoting the differentiation of immune-like cells towards an anti-inflammatory profile.
Intrathecal IL-1RA administration and the elevation of IL-10 and IL-4 levels in spinal cord result in attenuated nociceptive hypersensitivity associated to gliosis (Watkins et al., 1997; Leger et al., 2011).

3.2.2 Pharmacological consideration: present and future

Treatment of neuropathic pain is challenging. Compared with patients with non neuropathic chronic pain, patients with neuropathic pain seem to have higher average pain scores; to require more medications; and to report less pain relief with treatment (Torrance et al., 2007).
Pain processing involves multi-pathways and dynamic systems both in the PNS and in the CNS, which can be potential pharmacological targets for analgesia. Some of these are already used in clinic by existing analgesics (Farquhar-Smith, 2008).
Opioids are considered the most powerful analgesics; their efficacy can be explained by the large distribution of opioids receptors in the spinal cord and in areas of brain associated to descending pain modulatory pathways as locus coeruleus and PAG. Thus, opioids induce analgesia acting both on pain transmission and perception. Their use for the long-term treatment of chronic pain is controversial for many reasons like development of psychological addiction and side effects such as sedation, dizziness, nausea, vomiting, respiratory depression and tolerance (Benyamin et al., 2008).
Since most of pain inhibitory pathways are noradrenaline/serotonin-based it is not difficult to understand why the antidepressants (inhibitor of noradrenaline/serotonine re-uptake) may be effective in controlling pain. Side-effects (which commonly limit their use) include sedation, dry mouth, constipation, urinary retention and cardiac side-effects that may preclude the use of these drugs in patients with cardiac conduction disturbances or recent infarction (Ryder and Stannard, 2005).

Anticonvulsants, such as gabapentin and pregabalin, induce analgesia acting on neuron excitability by blocking of sodium and calcium channels. Side-effects of the antiepileptic drugs are usually those affecting the CNS, gastrointestinal and haematological systems. Non-steroidal anti-inflammatory drugs (NSAIDs), that prevent the formation of prostaglandins by inhibiting cyclooxygenase (COX), are generally not efficacious in neuropathic pain treatment.
The fundamental role of NMDA receptor in central sensitization makes it a potentially therapeutic analgesic target although, to date, only few NMDAR antagonists are available for clinical use.
Moreover, considering the key role of the neuroimmune interface in chronic pain there is great interest in targeting immune and glia components to manage painful conditions. Several promising strategies to target the neuroimmune interface include the direct inhibition of pro-inflammatory signaling, stimulation of local protective anti-inflammatory mechanisms, inhibition of specific immune mediators and the antagonism of specific cytokine and chemokine receptors (Grace et al., 2014).
In this direction, a better knowledge of the mechanisms underlying neuropathic pain could lead to the identification of novel promising targets for the development of more efficacious analgesic therapy.

3.3 Chemotherapy-induced peripheral neuropathy

Chemotherapy-induced peripheral neuropathy (CIPN) is a damage to the peripheral nervous system caused by the exposure to a neurotoxic chemotherapeutic agent. It is one of the main frequent dose-limiting side effect of several first-line antineoplastic agents (Table 1) such as vinca alkaloids, proteasome inhibitors, platinum based compounds and taxanes (Malacrida et al., 2019; Colvin et al., 2019).
Given the prevalence of the common cancers (e.g. breast, ovarian, colorectal) that these chemotherapeutics counteract, CIPN affects several million patients worldwide every year. The prevalence of CIPN ranges from 10 to 100% depending on different factors, such as the agent or drug combination administered, the dosing regimen, the methods of pain assessment and the presence of predisposing genetic factors or history of neuropathy (i.e. diabetes or obesity) before starting chemotherapy, impaired renal function with reduced creatinine clearance, and a history of smoking and chronic alcohol use (Flatters et al., 2017). This neuropathic pain syndrome interferes with optimal treatment of cancer disease since it often leads to the need for dose reduction, treatment delays and even premature cessation of chemotherapy, resulting in long-term debilitating effects that can cause increased morbidity and decreased quality of life (Brewer et al., 2016). The main sensory symptoms commonly reported by patients include paresthesia, dysesthesia, allodynia, hyperalgesia, hypoalgesia or pain that is burning, shooting or electric-shock-like (Boyette-Davis et al., 2015). CIPN has a typical “stocking and glove” distribution in the feet and hands, respectively, due to the vulnerability of the longest axons, but then the pain can expand all over the body; moreover painful symptoms may appear after treatment interruption, a phenomenon known as “coasting” (Quasthoff and Hartung, 2002). Furthermore, although slow recovery of peripheral nerve damage may occur in patients with CIPN, this is not always the case and therefore pain may also persist for months or years after the cessation of chemotherapy. Therefore, patients may well be cancer-free, but suffering a debilitating neuropathy evoked by their cancer treatment. This condition significantly impairs quality of life in several ways; in fact many patients report poorer quality of life related to alterations in lifestyle, increased need for assistance from others and changes in emotional cognitive and social function (Boyette-Davis et al., 2018). This neuropathic pain syndrome is challenging and complex and, up to now, there are no effective preventive or therapeutic treatments. Several drugs such as gabapentin and opiates are used to provide symptomatic relief but they lack efficacy and/or have unacceptable side-effects such as addiction development, dizziness and nausea (Balayssac et al., 2005), therefore there is a large medical need for novel, well-tolerated analgesic agents able to improve relief of CIPN.

Chemotherapy Agent	Antineoplastic Mechanisms	CIPN Pathophysiology
Taxanes	Microtubule damage that impairs mitotic spindle formation in cancer cells	Activation of caspases, oxidative stress on peripheral neuronal and non-neuronal cells, mitotoxicity, inhibition of anterograde fast axonal transport, prevention of microtubule disassembly, alteration of both activity and expression of voltage-gated ion channels in the DRG; immune activation in the DRG and peripheral nerves, and microglial activation in the spinal cord; TRP upregulation in DRG.
Vinca alkaloids	Binding to free tubulin dimers close to the GTP-binding sites and induction of cell death by inhibition of microtubule assembly.	Increase of microtubule depolymerization and inhibition of the hydrolysis of GTP; membrane excitability, inflammation, axonal transport impairment; mitochondria and glial function alterations; differential expression of voltage-gated ion channels, alteration of neurotransmission, impairment of axonal transport, increased production and release of proinflammatory cytokine and chemokines.
Eribulin	Suppression of microtubule dynamic instability at low concentration and promotion of microtubule disassembly at high concentration.	Reduction of anterograde fast axonal transport; reduction of kinesin-dependent transport in axon.
Epothilones	Stabilization of microtubules, leading to apoptosis in cancer cells.	Microtubule stabilization; reduction of kinesin-dependent transport.
Platinum compounds	Platinum-DNA adduct formation, alterations in transmembrane receptors and channels that lead to cell cycle arrest and apoptosis.	Accumulation of platinum atom in the DRG sensory neurons, nuclear and mitochondrial DNA damage, oxidative stress and channellopathy; TRP channels affected; axonal transport impairment; activation of caspases and protein kinase, glial cell activation, increased production and release of proinflammatory cytokine and chemokines.
Proteasome inhibitors	Inhibition of proteasome activity, which results in protein aggregate accumulation in tumor cells, cell cycle arrest and apoptosis.	Protein aggregate accumulation in soma neurons, alteration of physiological turnover of axonal proteins, axonal transport impairment; damage to neuronal mitochondria and organelles and Schwann cell microtubule stabilization, oxidative stress; activation of TRP channels; inflammation and glial cell activation; alterations of neurotransmission.
Thalidomide	Immunomodulation and antiangiogenic effects, down-regulation of tumor necrosis factor alpha (TNFα).	Capillary damage due to its antiangiogenic activity in nerve fibers; downregulation of TNFα and inhibition of NF-κB that interferes with NGF activity.

Table 1| Summary of antineoplastic and neurotoxic mechanisms in CIPN.

Table edited by Malacrida et al., 2019

3.3.1 Pathogenesis of CIPN

Most chemotherapeutic agents do not, or just in small part, penetrate the blood-brain barrier (BBB). On the other hand, they can easily cross the blood-nerve barrier (BNB) and they accumulate in dorsal root ganglia and peripheral nerves (Montague and Malcangio, 2017).

Penetration of the BNB can be attributed to both the dense vascularisation, the presence of fenestrated capillaries in the DRG and to relatively low levels of P-glycoprotein transporter activity, which limits the efficiency of toxin removal (Balayssac et al., 2005) . As well as the intrusion of toxins, immune cells also infiltrate into peripheral nerves and dorsal root ganglia. Therefore, the peripheral nervous system is considerably more susceptible than the central nervous system to chemotherapy related toxicity.

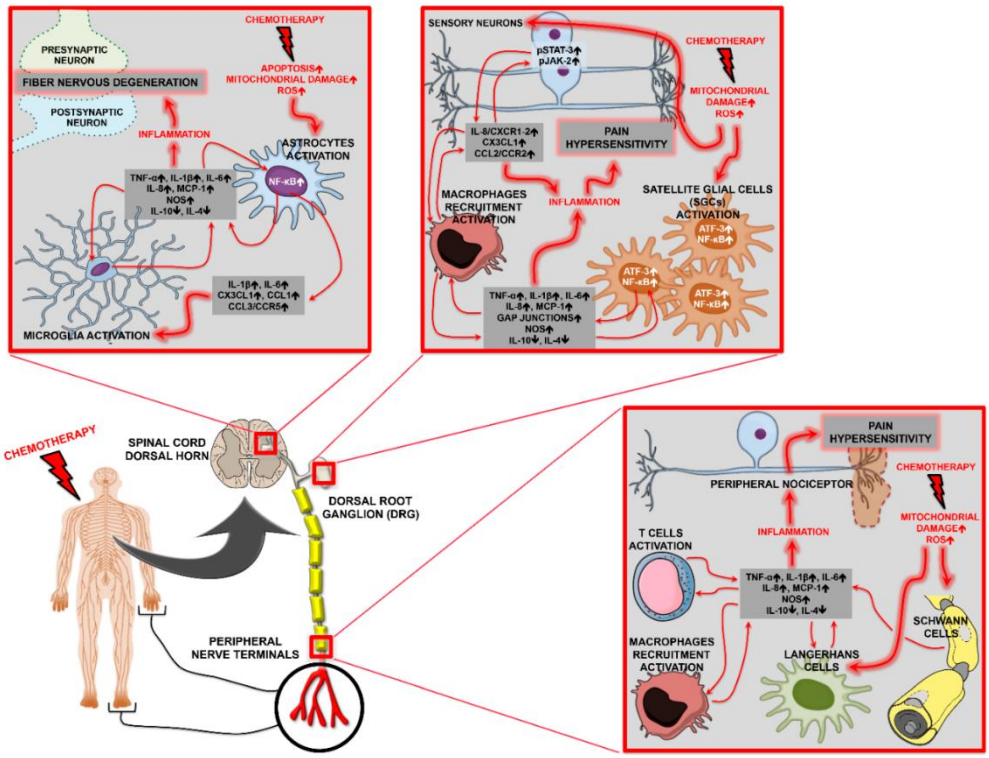


Image 6 | Effects of chemotherapeutic agents on nervous tissues.
Image from Brandolini et al., 2019.

- Structural changes in peripheral nerves

Chemotherapeutic agents may induce peripheral nerve degeneration or small fiber neuropathy, which is now generally accepted as a mechanism supporting the development of CIPN.

The different nerve fibers present in the peripheral nerves show a different sensitivity to the neurotoxic effects of antineoplastic agents: the longest nerves have the higher susceptibility (Wilkes, 2007; Gutierrez-Gutierrez 2010), that could be explained by their higher metabolic requirements.

Even if myelinated fibers are preferentially damaged by chemotherapeutic drugs (Cata et al., 2006), the extent to which demyelination is a key pathological event in CIPN is still unclear. It was shown by X-ray diffraction that no structural alterations of the myelin structure was present in the sciatic and optic nerves of rat subjected to chemotherapy with cisplatin, paclitaxel or bortezomib (Gilardini et al., 2012). However, higher doses of bortezomib were linked to an increased risk of peripheral nerve degeneration and possibly demyelination in contrast to lower dosage that were still able to induce neuropathy (Zheng et al., 2012).

Unmyelinated fibers and nerve terminal arbor are the major sites of chemotherapy neurotoxicity (Grisold et al., 2012), in fact intraepidermal nerve fiber (IENF) loss or terminal arbor degeneration has been proposed as a common lesion characterizing several toxic neuropathies (Bennet et al., 2011). A significant IENF decrease and axons degeneration was observed in some experimental models of CIPN due to the chronic administration of paclitaxel (Xiao et al., 2011, Authier et al., 2000), bortezomib (Meregalli et al.,

2010) and cisplatin (Arrieta et al., 2011). Furthermore, also in clinics, IENFs loss is commonly detected in CIPN patients (Boyette-Davis et al., 2011; Giannocaro et al., 2011). Interestingly, both experimental models and clinics show that the extent to which peripheral nerve axons are damaged by chemotherapy appear to be directly related to the dosage regimen.
The precise causes of IENF degeneration and abnormal spontaneous discharge of primary afferent nerve fibers in experimental models of CIPN may be related to mitochondrial dysfunction and the subsequent energy deficit (Boyette-Davis and Dougherty 2011; Xiao et al., 2011).

- Mitochondrial dysfunction

Over the last years, mitochondrial dysfunction was identified as a significant contributory factor in CIPN. Changes in mitochondrial localization, altered fission/fusion rates and changes in mitochondrial membrane permeation have all been investigated after neuronal exposure to antineoplastic agents. These alterations are usually associated to changes in calcium handling, release of cytochrome *c*, mitochondrial DNA damage and enhanced production of ROS, which can all contribute to neurotoxicity.
It has been demonstrated that paclitaxel and bortezomib induce mitochondrial swelling within sensory neuronal axons, soma and Schwann cells (Zheng et al., 2012; Flatters and Bennet, 2006). Furthermore, different studies showed that paclitaxel as well as oxaliplatin promote mitochondrial permeability leading to a depolarization, theoretically resulting in a decrease production of ATP for the neurons and enhancing the production of ROS (Batandier et al., 2004).

- Oxidative stress

Chemotherapeutics have been shown to enhance the production of both reactive oxygen (ROS) and reactive nitrogen (RNS) species by neurons, supporting satellite cells and infiltrating inflammatory cells (Jiang et al., 2008). The generation of nitroxidative stress in DRG sensory neurons as well as in the spinal cord has been shown to enhance the sensitivity of nociceptive neurons. Nitroxidative stress can compromise the survival and function of sensory neurons and their supporting cells by causing DNA damage, nerve fiber demyelination, mitochondrial damage and dysfunction, activation of signal transduction pathways and neuronal death via apoptosis. It is known from the 1990s that ROS/RNS are involved in neuropathic conditions (Tal, 1996) because of their effects on neuronal excitability. It was recently demonstrated in different *in vivo* models how oxidative stress has a role in CIPN, increased RNS production was found in the spinal cord of paclitaxel treated rats (Doyle et al, 2012) and in the lumbar DRG of oxaliplatin-treated mice (Toyama et al., 2014). The use of pharmacological reagents that scavenge ROS was tested in different CIPN models. PBN, a non specific ROS scavenger, inhibited the development of paclitaxel-induced mechanical hypersensitivity and reversed established mechanical and cold hypersensitivity due to paclitaxel (Fidanboylu et al., 2011) or bortezomib (Duggett et al., 2017). Collectively these experimental preclinical studies suggest that mitochondrial ROS/RNS is at the basis of the development and maintenance of CIPN. However, there are concerns about the development of antioxidant therapies to treat the pathology, because ROS and RNS production of has been shown to be fundamental for the anticancer effect of chemotherapy (Alexandre et al., 2006).

- Ion channels alterations

Changes in both voltage- and ligand-gated ion channels have been shown to play a role in nociceptive behaviour alterations typical of CIPN. Sodium-, potassium-, calcium- and Transient Receptor Potential (TRP) channels, mediate different modalities of CIPN even if their precise involvement is still unclear.
Changes in the behaviour of voltage-gated sodium channels have been found in CIPN. For example, a major metabolite of oxaliplatin, oxalate, produce prolonged opening of voltage-gated Na+ channels, leading to

altered thresholds and ectopic firing in diverse neurons (Webster et al., 2005). Interestingly, voltage-gated sodium channel blockers, such as the anticonvulsant carbamazepine, have shown some effect in treating neuropathy in patients (Grothey, 2005), although not all the clinical studies supported this positive result (Lehky et al., 2004)

Alterations in K+ channels function have been detected at several levels of the nervous system in CIPN models. These channels result down-regulated in primary afferent neurones of rats treated with oxaliplatin or paclitaxel (Zhang et al., 2014). In support of these findings, the Kv7 channels activator retigabine has been found to be effective in a mouse model of cisplatin neuropathy (Nodera et al., 2011).

Another cause of ion channels dysfunction, specifically following treatment with oxaliplatin, is the chelation of the extracellular calcium and magnesium by oxalate, a metabolic product of oxaliplatin. Voltage-gated calcium channels are key regulators of synaptic transmission and they are implicated in the hyper-excitability observed in CIPN. Paclitaxel treatment induced an increase in calcium channel mRNA in DRG of treated mice. Consistently with these findings, paclitaxel treatment was shown to alter calcium metabolism in primary afferent fibers, which was reversed by the treatment with the potassium channel opener minoxidil (Chen et al.,2017), while also ameliorating the behavioural signs of CIPN.

TRP channels are a large family of nonselective cation channels. In particular, the TRP vanilloid (TRPV) family is the most widely studied in relation to pain and CIPN. TRPV1, the capsaicin receptor, is located on a wide subset of nociceptors and plays an important role in sensory transduction and primary afferent sensitization. Recent evidence shows that the activation of TRPV1 during chemotherapy sensitizes pain pathways. The expression of this receptor is in fact upregulated in rat DRG following paclitaxel administration, at a time point that corresponds to the onset of thermal hyperalgesia. Furthermore the use of TRPV1 antagonists in a rodent model was able to reduce heat hypersensitivity (Hara et al., 2013). The increased TRPV1 expression is not linked to the specific chemotherapeutic agent, in fact also bortezomib (Quartu et al., 2014) and cisplatin (Guindon et al., 2013) induced its upregulation in different nervous tissues (i.e. DRG and spinal cord).

TRP ankyrin 1 (TRPA1) is often colocalized with TRPV1 and can be activated by diverse noxious chemicals and by cold temperatures (≤17°C). Oxaliplatin, paclitaxel and vincristine produces a robust sensitivity to cold in most CIPN patients, and based on animal studies indicating that TRPA1 channels respond to noxious cold, it was assumed that this channel was responsible for this effect (Tseng et al., 2011; Materazzi et al., 2012). There is also the possibility that TRPA1 may be involved in the induction of long-lasting sensitization of nociceptors during chemotherapy.

Also the transient receptor potential melastatin 8 (TRPM8) channel is activated by mild cold stimuli between 25 and 28°C and chemically by menthol and has been implicated as analgesic when activated in some nerve injury models (Proudfoot et al., 2006) and could be involved also in CIPN.

- Neurotransmitters

Several studies have demonstrated the presence of alterations in processes involved in neurotransmission following chemotherapy treatment.

There are evidences that serotonin (5HT) receptor changes are involved in CIPN. Specifically, mice lacking 5HT receptors (2A) show protection against vincristine-induced mechanical hyperalgesia and wildtype rats exposed to vincristine had an increase expression of this receptor at the level of the spinal cord (Thibault et al., 2008).

Glutamate signaling is also altered in CIPN, in fact it has been shown that glutamate levels increase following oxaliplatin exposure in rats, and the use of a polyamine-deficient diet to modify glutamate levels, and subsequent NR2B activation, prevented oxaliplatin-induced hypersensitivity to cold and mechanical stimulation (Ferrier et al., 2013). Involvement of glutamate is not limited to oxaliplatin treatment, in fact *in*

vitro studies using rat spinal astrocytes show a decrease in glutamate transporter GLAST after treatment with bortezomib, paclitaxel and vincristine (Cata et al., 2006; Robinson and Dougherty et al., 2014). Given that spinal neurons treated with different agents show pronounced discharges this phenomenon appears to be related to the downregulation of glutamate transporters.
Changes to other neurotransmitter systems have been also observed in animal models of CIPN, including decreased spinal mu-opioid receptor activity in DRG and spinal cord of vincristine-treated mice (Yang et al., 2014). In these animals, vincristine-induced allodynia was associated with decrease endogenous activity on mu-opioid receptors (Yang et al., 2014), but it is important to notice that exogenous administration of mu-opioid receptor agonists (i.e. morphine and other opioid-based analgesics) do not address the array of CIPN symptoms or even fully control chemotherapy pain clinically (Boyette-Davis et al., 2013).

- Neuroinflammation

Chemotherapeutics exert systemic immunomodulatory effects. At therapeutic doses, the effect is often transiently immunosuppressive due to the cytotoxic activity targeting rapidly dividing cells. However, there are increasing evidences suggesting that many antineoplastic agents can stimulate the immune system.
The peripheral nervous system contains several types of glial cells. Neuronal cell bodies in the DRG are covered by flattened sheet-like cells, satellite glial cells (SGCs), whereas axon in the nerve trunks are ensheathed by non-myelinating or myelinating Schwann cells. These resident cells respond to chemotherapy by undergoing phenotypic changes and stimulating the secretion of different mediators that enhance neuronal excitability and may generate pain hypersensitivity (Carella et al., 2000; Warwick et al., 2013). In particular, during the development of CIPN there are several pathological changes in SGCs such as hypertrophy, increased expression of the marker of cell injury ATF3 (activating transcription factor-3) and mitochondrial and endoplasmic reticulum enlargement. Furthermore, some evidences obtained in rodent studies show how satellite cells may undergo apoptosis and thereby contribute to CIPN worsening, either by secretion of apoptotic signaling or gap junction coupling. Also Schwann cells start to express ATF3, show hypertrophy, presence of large cytoplasmic vacuoles and myelin ovoids.
Not only resident cells participate in CIPN development but also infiltration of monocytes into the peripheral nervous tissues, DRG and sciatic nerve, where they differentiate into inflammatory macrophages, has been observed in several experimental models of CIPN and corresponds with model-associated pain (Montague and Malcangio, 2017). Paclitaxel treated rats, for example, show an increase of macrophage number at the level of DRG, which was concurrent with the development of cold hyperalgesia and mechanical hypersensitivity (Peters et al., 2007). Involvement of these cells in the pathology was sustained also by pharmacological studies, where the use of minocycline (inhibits monocyte/macrophage infiltration) or the depletion of macrophages (using liposome-incapsulated clodronate, LCL) reduced, respectively, oxaliplatin and paclitaxel induced pain (Liu et al., 2010; Boyette-Davis and Dougherty, 2011). The association between monocytes/macrophages and painful symptoms was demonstrated also in a vincristine model of CIPN. Old and collegues (2014) demonstrated how already 24h after a single VCR dose there is a significant increase of macrophages in the DRG and sciatic nerve, which remain elevated also few weeks after, treatment interruption. Interestingly, after a few weeks from the end of the chemotherapy schedule, when pain disappears there is a concurrent reduction in macrophages number in the analyzed nervous tissues.
In the central nervous system there are two general classes of glia: astrocytes and oligodendrocytes and the microglia, which consist of macrophage-like glial cells. The involvement of microglia and astrocytes, through their inflammatory effects, in the development of neuropathic pain is well-known (Watkins et al., 2002). In fact central glial cells secrete pro- and anti- inflammatory cytokines and chemokines, ATP and also other small inflammatory molecules able to modulate neuronal activity (Ji et al., 2013). The majority of

CIPN studies demonstrate a long-term increase in spinal astrocytes activation, while microglial activation at the level of the spinal cord is still debated, with some evidences for no activation, or just a transient increase (Di Cesare Mannelli et al., 2014; Zhang et al., 2013). Even if there are still discrepancies among these studies, taken together these preclinical studies provide evidence that astrocytes and, in many case, microglia activation occurs within the CNS following treatment with antineoplastic agents.

The involvement of cytokines and chemokines in the pathological processes of CIPN was demonstrated by several preclinical studies. Chemotherapeutic exposure significantly enhances cytokine release. In particular, administration of vincristine (Weintraub et al., 1996), paclitaxel (Grothey et al., 2005), cisplatin (Basu et al., 1992) leads to an increase in the expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, accompanied by a reduction in the expression of anti-inflammatory cytokines (IL-10 and IL-4) within the DRG and spinal cord was observed. These cytokines can bind to receptors on neurons and glial cells to enhance activity in pain pathways and, in turn, increase expression of cytokines in the same cells, thus leading to a self-sustaining cycle (Ledeboer et al., 2007).

The release of cytokines from cells following chemotherapy may be related to the ability of antineoplastic drugs to activate the Toll-like receptor (TLR) family, especially TLR4 (Byrd-Leifer et al., 2001). These receptors have been recently shown to be involved not only in innate immunity, but also in pain processes. In fact, increased levels of TLR4 are present in the DRG of rats displaying paclitaxel-induced hyperalgesia and the use of TLR4 antagonist was able to significantly ameliorate the pathological condition (Li et al., 2014). Similar results were obtained by knocking out the receptor in oxaliplatin-treated mice (Park et al., 2014).

Like cytokines, also chemokines play a significant role in CIPN, by promoting the activation and infiltration of macrophages and glial cells. Several studies show an upregulation of the chemokine (C-C motif) ligand 2 (CCL2 or MCP-1) and its receptor CCR2 in DRG of models of CIPN induced by paclitaxel (Warwick et al., 2013; Park et al., 2014; Zhang et al., 2013), oxaliplatin (Zhang et al., 2016) and bortezomib (Liu et al., 2016). Neutralizing the chemokine resulted in all cases in a decrease pain responding.

3.3.2 Bortezomib

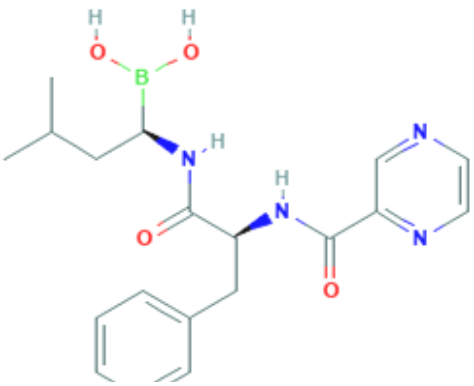


Image 7| Bortezomib chemical structure
Image from Pubchem

Bortezomib, BTZ, (Velcade®, formerly known as PS341) is the first of a new family of chemotherapeutics, known as proteasome inhibitors, approved by the US FDA in 2003.

The chemical name for bortezomib, the monomeric boronic acid, is [(1R)-3-methyl-1-[[(2S)-1- 15 oxo-3-phenyl-2-[(pyrazinylcarbonyl) amino]propyl]amino]butyl] boronic acid. Bortezomib chemical structure is shown in image 7.

The molecular formula is C19H25BN4O4 and the molecular weight is 384.24.

BTZ is currently used as a first-line agent in treatment of newly diagnosed and relapsed multiple myeloma (Engelhardt et al., 2010), with further trials showing effectiveness against non-Hodgkin's lymphoma (Bose et al., 2014) and solid carcinomas, including renal, prostate and ovarian tumours (Cusack, 2003; Huang et al., 2014).

The ubiquitin-proteasome pathway is the major proteolytic system in the cytosol and nucleus of all eukaryotic cells and it plays an essential role in regulating homeostatic and other cellular events including those involved in tumorigenesis.

Therefore, targeting the regulation of protein production and destruction, particularly the factors that determine proliferation and other hallmark characteristics of cancer cells, has been a major focus of cancer research.

Recently, it has become widely appreciated that the orderly destruction of cell cycle regulatory proteins is critical to the control of cellular processes associated with tumors. The ubiquitin-proteasome pathway (UPP), which processes more than 80% of all cellular proteins, is the principal mechanism for degradation of proteins, including those involved in cell cycle regulation (Adams, 2002). Accordingly, the proteasome has emerged as an attractive target for cancer therapy.

Proteins are targeted for recognition and for subsequent degradation by the proteasome via the attachment of multiple ubiquitin (small 8kDa protein) molecules. The 26S proteasome is a 2,400 kDa multisubunit hollow cylindrical complex composed by a 20s catalytic core (the 20s proteasome) capped at one or both ends by a 19S regulatory component. The 19s subunit recognizes and binds the polyubiquitinated protein and cleaves the ubiquitin chain from the protein substrate.

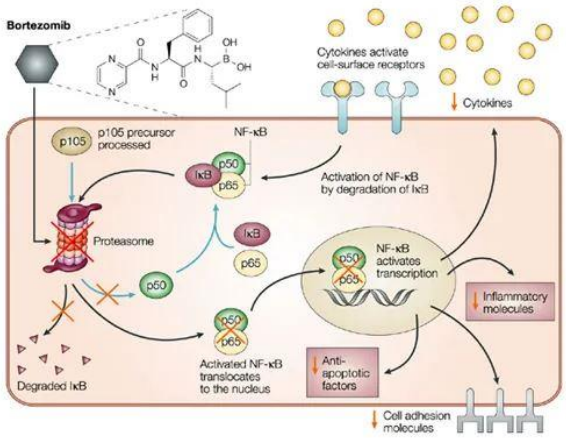


Image 8| Bortezomib mechanism.
Image from Paramore & Frantz, 2003.

The protein is then unfolded and fed into the 20s core, while the ubiquitin molecules are recycled. The 20s core is composed of 4 stacked rings: 2 outer rings (α rings) and 2 internal rings, termed β rings, in which proteolysis occurs. Each β ring consists of 7 subunits containing 3 active enzymatic sites termed trypsin-like, chymotrypsin-like, and post-glutamyl peptide hydrolase-like (caspase-like), after enzymes that show similar activity or specificity. Within the 20S core, proteins are progressively degraded to small, 3- to 25-aminoacid peptides (Adams, 2003). Although the proteasome has multiple active sites, inhibition of all of them is not required to significantly reduce protein breakdown.

Bortezomib is a modified boronic acid dipeptide which reversibly binds the β5 subunit and inhibits the 20s catalytic subunit (chymotrypsin-like) of the 26S proteasome (Adams, 2002). Since as previously mentioned, multiple proteins are degraded by the proteasome, multiple cellular processes are affected by proteasome inhibition leading to the disruption various cell signaling pathways, thereby leading to cell cycle arrest, apoptosis and inhibition of angiogenesis (Argyriou et al, 2017). Therefore, the activity of bortezomib in different cancers probably involves a variety of molecular mechanisms. Proteasome inhibition has been shown to interrupt critical cell pathways, including the ubiquitin-proteasome pathway and p53, JNK and NFκB activity, to induce apoptosis (Hideshima et al., 2003; Voorhees et al., 2003). Malignant cells are more sensitive to proteasome inhibition, undergoing apoptosis more readily than healthy cells. Bortezomib can indirectly polymerise tubulin, causing microtubule stabilization, axonal transport inhibition and G_2M phase cell cycle arrest (Poruchynsky et al., 2008; Rapino et al., 2013; Staff et al., 2013).

Clinical BTZ pharmacology is poor, the pharmacokinetics of bortezomib have not been fully characterized in multiple myeloma patients. It was demonstrated that after intravenous (i.v.) bolus administration, bortezomib quickly distributes from the plasma into peripheral tissues (Romano et al., 2013). The distribution half-life is less than 10 minutes, followed by a long elimination half-life (> 40 hours) (Nix et al., 2003). *In vitro* studies with human liver microsomes and human cDNA-expressed cytochrome P450 isozymes indicate that bortezomib is primarily oxidatively metabolized via cytochrome P450 enzymes 3A4, 2C19, and 1A2. Bortezomib metabolism by CYP 2D6 and 2C9 enzymes is minor. The major metabolic

pathway is deboronation to form 2 deboronated metabolites that subsequently undergo hydroxylation to several metabolites. Deboronated bortezomib metabolites are inactive as 26S proteasome inhibitors. Pooled plasma data at 10 min and 30 min after dosing indicate that the plasma levels of metabolites are low compared to the parent drug. The pathways of elimination of bortezomib have not been characterized yet.

3.3.3 Vincristine

Vinca alkaloids (VAs) are a subset of anti-mitotic and anti-microtuble agents originally isolated from the leaves of the Madagascar periwinkle, *Catharanthus roseus* (Vinca rosea L.).

The vinca alkaloid drug family is composed by: vincristine, vinblastine, vinorelbine, vindesine and vinflunine, of which the latter three are all semisynthetic derivatives of vinblastine (Moudi et al., 2013). Among vinca alkaloids, vincristine sulfate (also known as Leurocristine and marketed under the brand name Oncovin) was first isolated in 1961 and it was approved by US FDA in the form of "Oncovin" in July 1963. It was primarily used to treat acute lymphoblastic leukaemias and lymphomas.

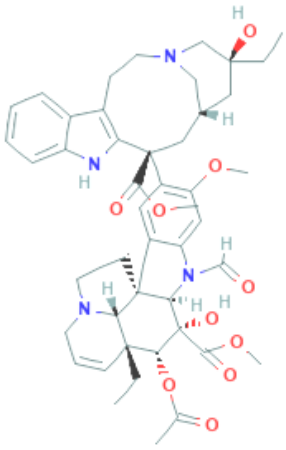


Image 9| Vincristine chemical structure. *Image from Pubmed*

The molecular structure of vincristine is asymmetrical and dimeric, composed of a dihydroindole nucleus, vindoline, linked by a carbon-carbon bond to an indole nucleus, catharanthine. Vincristine sulphate is the 1:1 sulphate salt of the alkaloid extracted from *Catharanthus roseus*. The empirical formula of vincristine sulphate is C46H56N4O10H2SO4. The molecular weight is 923.1 kDa (Gidding et al., 1999).

Vincristine exerts many effects on cells, using a variety of mechanisms, the most established way by which it inhibits tumor growth is its interference with the mitotic splindle microtubules that leads to apoptosis (Owellen et al., 1976).

Structurally, microtubules are polymeric heterodimers composed by α and β-tubulin subunits. These α/β-tubulin dimers polymerize end-to-end into linear protofilaments that are adjacent to each other and roll to generate a single cylindrical microtubule, which can then be extended by the addition of further α/β-tubulin dimers. The biological function of microtubules is mostly determined by their polymerization dynamics that can be grouped into treadmilling and dynamic instability. Treadmilling implies that the addition of the nucleating filament and tubule dimers is more efficient to the (+) end than the (−) one. Conversely, dynamic instability is characterized by changes in the length of the microtubule structure, primarily at the (+) end and this process depends on the presence or absence of a GTP β-tubulin cap. Both α and β subunits of the tubulin dimers bind one molecule of GTP, but only the one associated with the β-subunit undergoes hydrolysis, and the resulting GDP can be exchanged for free GTP. The addition of a dimer to the (+) end of a protofilament triggers the hydrolysis of the GTP to GDP in the former terminal β-subunits. However, the β-tubulin in the newly added dimer contains GTP. Thus, each protofilament in a growing microtubule has mostly GDP-β-tubulin down its length and is capped by terminal dimers containing GTP-β-tubulin.

Microtubules control intracellular trafficking of proteins, organelles, vescicles and separate chromosomes during mitosis. These structures are also fundamental for cell proliferation, cell shape, migration, secretory processes, vascularisation and possibly signal transduction. In addition to microtubule-only functions, actin-tubulin interactions can control cell migration, wound healing, T cell immune response and growth of neuronal cones. Disruption of microtubules can alter the abundance of actin and intermediate filaments impacting cytoskeletal networks. However, there is no backup redundancy for microtubules within the

cellular cytoskeleton as they are the only structures able to segregate chromosomes or traffic cellular components (Gidding et al., 1999).

Vinca alkaloids constitute a valuable class of anti-cancer molecules with the ability to interact with tubulin dimers in 16–17 high-affinity binding sites presenting at the end of each microtubule and thus inhibit microtubule polymerization. The rapid and reversible binding to the microtubules inhibits cell mitosis through vinca alkaloids binding to tubulin proteins in the mitotic spindle, avoiding its polymerization into microtubules and the anaphase onset, therefore cells remain in a senescence-like G1 state or in a prolonged arrest state with subsequent rapidly dividing cells death (Martino et al., 2018).

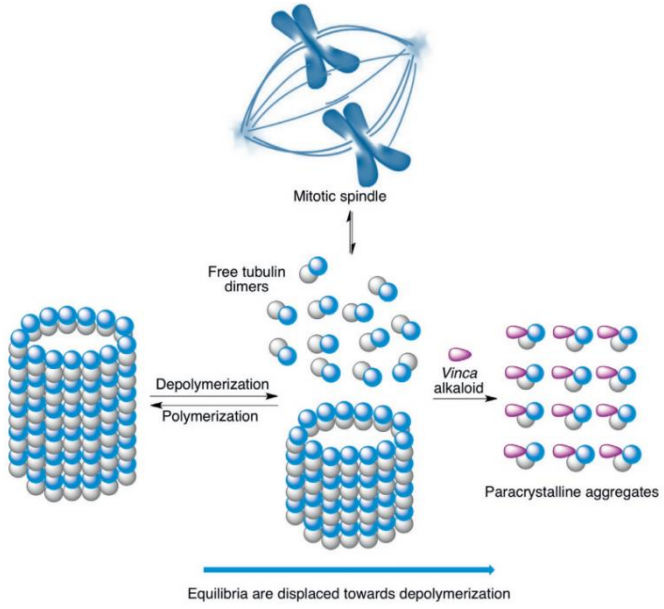


Image 10| Depolymerization of microtubules following binding of Vinca alkaloids.
Image from Avendaño & Menéndez, 2015.

Vinca alkaloids drugs are typically administered by intravenous bolus, but they can be given as a longer infusion to mantain high drug levels for a longer period of time. Clinical pharmacokinetic studies after continous infusion of vincristine show a wide interpatient variability which may be explained by differences in dosage regimens, age, haematological and biochemical characteristics, and concomitantly administered drugs.

Vincristine clearance from plasma is characterized by a rapid distribution stage and an uptake into peripheral tissues (such as pancreas, spleen, thyroid, adrenal, intestinal mucosa, lung, liver, kidney and bone marrow) and formed blood elements, which possess a high content in tubulin. On the other hand, animal studies show a low vincristine retention in brain, eye and fat. Vincristine concentrations measured in human cerebrospinal fluid are 20–30 times lower than concentrations found in plasma. Tissue distribution is stricktly related to the tubulin isotype composition of each tissue, from which VAs are deeply dependent, but also to vincristine lipophilicity. VCR terminal half-live ranges from 18 to 85 h, the drug is then excreted mainly via the bile and feces and minimally (from 1% to 30% of the total drug disposition) via the renal pathway in humans. Vincristine is excreted partly as unchanged parent drug, and partly as metabolites.

Details about Vincristine metabolism are still not known, it is believed that human liver cytochrome P450 3A is probably involved in the metabolic biotransformation of vinca alkaloids, since drugs (like erythromycin and cyclosporin A) which are metabolized by human cytochrome P450 3A competitively inhibit vinca alkaloid biotransformation. Moreover, pentobarbital, which induces hepatic cytochrome P450 activity, increases vincristine clearance. The extent to which these metabolites or degradation products have biological activity is yet unknown (Gidding et al., 1999).

3.4 Animal models of chemotherapy induced peripheral neuropathy

In the past years, new insights on the mechanisms underpinning the pathogenesis of CIPN have been made possible thanks to the advent of rodent models enabling new targets to be identified for use in pain therapeutics discovery programs.

The development of rodent models able to replicate the most common symptoms that are generally reported by patients is challenging because it is not possible to measure spontaneous pain (numbness, tingling and ongoing pain) which, in clinics, rely on verbal report from the patient. Thus, most of the preclinical testings have focused on measuring evoked pain-like behaviours, as has been the case for experimental studies involving other chronic pain models.
Most of the chemotherapeutics drugs known to cause peripheral neuropathy in patients have been used to develop rodent models of CIPN. Commonly used ones include platinum compounds, taxanes, proteasome inhibitors, vinca alkaloids and thalidomide (Höke et al., 2014). Rodent models of CIPN are generated using different dosing regimens and timing of administration, most of the models rely on intermittent systemic administration of the particular chemotherapeutic, in order to mimic the cycles of chemotherapy used in patients as opposed to daily dosing.
Usually, most models of CIPN involve only the administration of a given chemotherapeutic in the absence of tumour load. This is due to the fact that animal health must be considered when using rodent models of CIPN for ethical reasons and also for practical feasibility; in fact pain-like behaviours cannot be accurately assessed if rodents are ill as a result of systemic toxicity and thus lethargic/unresponsive to hind paw stimulation. In addition, as chemotherapy is often received after surgical removal of the tumour to eliminate possible micro-metastases, modelling CIPN through chemotherapy administration alone is a valid approach (Flatters et al., 2017).
Furthermore, mouse models of CIPN resemble the clinical situation, in fact these models usually display sensory symptoms such as mechanical allodynia, mechanical hyperalgesia, cold allodynia and, in some reports, heat hyperalgesia.

3.4.1 BTZ animal models

Since proteasome inhibitors are a fairly novel form of chemotherapy, the first attempts at modeling this type of CIPN were done in rats, followed by studies that tried to characterize a mouse model of BIPN (Bruna et al., 2010, Cavalletti et al., 2007, Meregalli et al., 2010). These initial studies established a dose of 0.3 mg/kg of bortezomib twice a week as an intolerable dose, resulting in death of female Wistar rats. A similar investigation using the same type of animals found that a dose of 0.2 mg/kg 3 times per week for 4 weeks was tolerable (Carozzi et al. 2010).
As models of bortezomib-induced neuropathy are developed, they lack consistencies in mode of administration (intravenous or intraperitoneal route) and dosing schedule. Although there is a trend of using adult, female Wistar rats, recent studies varied in almost every aspect of their models (Cavaletti et al. 2007; Meregalli et al. 2010; Meregalli et al. 2012; Zheng et al. 2012). In these studies, they administered bortezomib 0.2 mg/kg with intraperitoneal injections to adult, female Sprague-Dawley rats once a day for 5 consecutive days with no deaths (Zheng et al. 2012). The other study involved the same dose (0.2 mg/kg) of bortezomib administration to female, adult Wistar rats but with intravenous injections 3 times a week for 8 consecutive weeks, which resulted in a much higher cumulative dose (Meregalli et al. 2012).
Similar to the rat models, although fewer studies have been completed in mice, there is no standard mouse model. One study used 2.5-month-old Swiss OF1 female mice and administered 1 mg/kg bortezomib by subcutaneous injection twice a week for 6 weeks; mild to moderate sensory neuropathy was reported (Bruna et al. 2010). Another study attempted to evoke bortezomib-induced neuropathy using a single dose (0.2 mg/kg, 0.5 mg/kg, 1 mg/kg) by intraperitoneal injection in C57BL/6 mice and found a dose-dependent effect lasting up to 11 days after administration (Trevisan et al. 2013).
A more recent study used BTZ intraperitoneally injected at the dose of 0.4mg/kg, 3 times a week for 4 consecutive weeks: this treatment schedule was able to induce a detectable neuropathic phenotype while at the same time limiting systemic side-effects to a minimum (Bohemerle et al., 2014).

3.4.2 VCR animal models

Also for vincristine the first animal models of vincristine-induced peripheral neuropathy (VIPN) were completed using rats (Aley et al., 1996; Boyle et al., 1996). Initial rat models of VIPN followed similar doses and schedules of daily injections, 5 consecutive days a week for 2 successive weeks, which was similar to what was done in the clinic. These early studies established a dose of 0.1mg/kg injection as sufficient to induce clinically relevant symptoms in rats. Variations were however present, especially regarding the route of administration (intravenous or intraperitoneal) and, in most recent studies, doses vary between 0.5mg/kg and 0.15mg/kg with some laboratories administering over the course of 2 weeks (Jaggi and Singh 2012; Sweitzer et al., 2006; Wala et al., 2012).

When mice are used as a model of VIPN, there is even more heterogeneity in the VCR doses; the doses used range from 0.1mg/kg to 2mg/kg (Apfel et al., 1993; Saika et al., 2009). Also the schedules used for administering VCR show a great variation. In some cases, the same clinically relevant schedule used in rat models (treat for 5 days, skip 2 days, treat for another 5 days) is used (Uceyler et al. 2006). Other studies deviate from this schedule, dosing twice each week for a period of 8 to 10 weeks or daily for 7,10 or even 14 days (Apfel et al. 1993; Contreras et al. 1997; Saika et al. 2009; Kiguchi et al., 2008).

3.5 *In vitro* models of CIPN

To accurately study the intracellular signaling mechanisms by which antineoplastic agents alter the sensitivity of sensory neurons, researchers have included in their studies *in vitro* models of CIPN represented by primary cultures of dorsal root ganglia. These cell cultures, composed by neurons, satellite glial cells and a moderate number of macrophages (Leisengang et al., 2018) provide a tighly controlled system which allows relatively easy manipulation of protein expression and function using both genetic and pharmacological tools. For example, McDonald and collegues (2002), using short-term cultures from DRG have shown that cisplatin-induced neurotoxicity involves the activation of apoptotic pathways, bax redistribution and cytochrome c release at the level of DRG neurons. Several researchers assessed the effect of chemotherapeutic drugs on electrical excitability, in particular it was observed an increase in the excitability of medium and large-size neurons isolated from paclitaxel-treated rats (Kawakami et al. 2012).The contribution of voltage-gated calcium current to this excitability was assessed in neurons collected from paclitaxel treated rats, it was shown how the chemotherapeutic drug enhanced voltage-dependent calcium current in both small- and medium-sized neurons (Kawakami et al., 2012). These data suggest a chemotherapy induced increase in the excitability of DRG sensory neurons.

Neurons treated *in vitro* with paclitaxel showed an increase in the TRPV1- and TRPA1- mediated release of calcitonin gene-related peptide (CGRP), while those treated with cisplatin are characterized by a decrease in the TRPV1-mediated CGRP release (Jiang et al., 2008). The different effects of the various classes of chemotherapeutics on the neuropeptide release suggest a different mechanism of action to elicit changes in neuronal sensitivity.

Since sprouting and elongation of axons and dendrites, at the right time and in the right direction, is at the basis of a proper neuronal connectivity and thus functionality, some studies focus on the morphological alterations of neurons due to chemotherapy. Neuronal cultures allow such investigations since neurons are able of reproducing *in vitro* the basic architectural rearrangements that are observed also *in situ* (da Silva and Dotti, 2002).

DRG cell cultures treated *in vitro* with cisplatin or paclitaxel show a a dose- and time- dependent fragmentation of neurites and enlarged neuronal cell bodies, which preceded the loss of neurites or cells (Guo et al., 2017). Also cells incubated with nanomolar concentrations of bortezomib showed shorter and thicker neurites, with swellings and unravelling microtubules (Alè et al., 2014). Interestingly bortezomib,

cisplatin, paclitaxel and vincristine caused these toxic effect even if they were used at concentrations three times lower the peak plasma concentrations observed clinically (Cmax). These effects were compared to drugs that have not been shown to cause CIPN, such as gefitinib and hydroxyurea, were without any statistically significant effects at concentration 4-fold of the Cmax (Guo et al., 2017).
Leisengang and collaborators (2018) analised the changes in satellite glial cells, they show how, also *in vitro*, these cells surround and cover the surface of neuronal cell bodies like under *in vivo* conditions. Those glial cells who did not cover the surface of neurons directly usually form pronounced cellular processes in culture. Treatment of these cell cultures with cisplatin induced a reduction in the number of both satellite cells and macrophages present in DRG primary cultures. The reduced number of these type of cells could impact on neuronal survival and functionality, becoming another mechanism at the basis of CIPN development.

3.6 The prokineticin system

Prokineticins (PKs) are a family of multifunctional chemokine-like small secreted peptides highly preserved throughout evolution, from invertebrates (crayfish, shrimp) to various species of mammals, including mice and humans.
The first members of this family to be discovered were VRPA or MIT1, a non-toxic constituent of the venom of the black Mamba snake (Joubert and Strydom, 1980) and Bv8 a small basic protein of 8 kDa isolated from the cutaneous secretion of the *Bombina variegata* frog (Mollay et al., 1999). Bv8 orthologs were then identified in the skin secretion of other amphibians but also in reptiles, fishes and mammals. In particular, in mammals two Bv8 homologs were identified: prokineticin 1 (PK1 or endocrine gland-derived vascular endothelial growth factor, EG-VEGF) and prokineticin 2 (PK2 or mammalian-Bv8) (Negri et al., 2007).
In the beginning, prokineticins were considered as potent agents mediating gastrointestinal motility and on the basis of this ubiquitous function present across different species, they were named “prokineticins” (peptides that cause gut movement). Later, it was shown that the same peptides were also able to promote angiogenesis in steroidogenic glands, hearth and reproductive organs (Ngan and Tam, 2008). Over the last decades it was demonstrated that this family of peptides mediate several biological functions such as neurogenesis, circadian rhythms regulation, nociception, hematopoiesis as well as inflammatory and immunomodulatory processes (Negri et al., 2007). Emerging evidences show that Prokineticins are implicated not only in pathologies of the nervous system but also in myocardial infarction, reproductive illness and tumorigenesis (Negri and Ferrara 2018; Ngan and Tam, 2008).

3.6.1 Prokineticins and their receptors: gene sequence, protein structure and anatomical localization

Mammalian prokineticins PK1 and PK2 are encoded by distinct genes with different chromosomal localization. PK1 gene (pk1) maps to regions of human chromosome 1p21.1 and mouse chromosome 3 and is organized in three exons encoding a mature form of 86 aminoacids (LeCouter et al., 2003a; Lin et al., 2002b), whereas PK2 gene (pk2) is located on human chromosome 3p21.1 and mouse chromosome 6 (Jilek et al., 2000) and consists of four exons which originate two mature proteins: PK2 of 81 aminoacids and a splice variant with 21 additional basic aminoacids, PK2L (102 aminoacids). This peptide is known to be cleaved by protease into a smaller active peptide called PK2Lβ (Chen et al., 2005). However, the biological function of the long isoform PK2L and of its smaller active peptide, PK2Lβ, has not been elucidated yet.

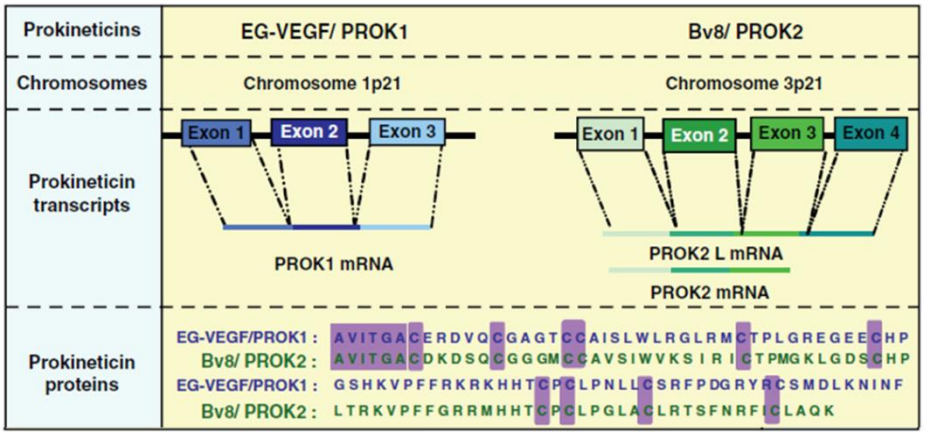


Image 11| Prokineticins.
Image edited by Brouillet et al., 2012

The genomic organization of PK1 and PK2 is similar. The first exon encodes for 19 residues and the first five aminoacids of the mature proteins corresponding to signal peptide and the conserved AVITGA sequence domain, respectively. The second encodes 42 aminoacids, including 6 of the ten cysteins characterizing the mature form of the proteins, while the third exon, which is absent in the genomic structure of PK1, encodes the 21 aminoacids insert that is instead present in an alternative mRNA of PK2. The remaining 34 aminoacids are encoded by last exon and include 4 of the ten cysteines of the secreted proteins (Negri et al., 2007; Martin et al., 2011).

Prokineticins and Bv8-related peptides share an high structural identity, particularly in the N-terminal and central regions. All these peptides in fact exhibit an identical amino terminal sequence, AVITGA (Ala, Val, Ile, Thr, Gly, Ala), which is essential for receptor recognition and therefore for their biological activity (Bullock et al., 2004; Negri et al., 2005). This is supported by the fact that mutations such as amino acidic deletions, insertions and substitution (Bullock et al., 2004), as well as proteolytic fragmentation of the conserved domain (Negri et al., 2005), result in partial or complete loss of the biological activity.

The presence of this peculiar highly preserved domain led Kaser and collegues (2003) to name this class of peptides the “AVIT proteins” in order to group in a unique family (the so called “AVIT family”) both the prokineticins and their non-mammalian orthologs.

Another common structural feature of these proteins is the presence of ten cysteine residues with identical spacing (Kaser et al., 2003) and a tryptophan residue in position 24 (Trp24).

The ten cysteines form five disulphide bridges conferring to the molecules a stable and compact three-dimensional structure which makes them highly resistant to protease degradation (Kaser et al., 2003). The N- and C- ends as well as some hydrophobic residues, including Trp24, are exposed on the surface, whereas the more charged residues are buried inside the molecule (Boisbouvier et al., 1998). One side of the roughly ellipsoid proteins has a positive net charge, whereas the opposite side is hydrophobic (Kaser et al., 2003).

PK1 and PK2 were identified in an wide array of organs and tissues such as brain, ovary, testis, placenta, adrenal cortex, intestinal tract, hearth and bone marrow (Ngan and Tam, 2008; Negri et al., 2009). Lymphoid organs, peripheral blood cells, synoviocytes and dendritic cells also constitutively express moderate levels of prokineticins (LeCouter et al., 2004; Martucci et al., 2006; Franchi et al., 2008).

PK1 is predominantly expressed in steroidogenic organs (LeCouter et al, 2001) whereas PK2 is mainly (but not exclusively) expressed in the nervous system and non-steroidgenic cells of testis (Ferrara et al, 2004; Cheng et al., 2005). While low expression of PK1 was detected in brain, PK2 is abundantly expressed in this organ, particularly in suprachiasmatic nucleus and the olfactory bulb (Cheng at al., 2002; Ng et al., 2005).

Few years after the discovery of prokineticins, three independent research groups identified in mammals two closely related G-protein coupled receptors for Bv8/PKs: prokineticin receptor 1 (PK-R1) and prokineticin receptor 2 (PK-R2) (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). Prokineticin receptors belong to the neuropeptide Y (NPY) receptor class, they show an overall identity in their

aminoacid sequences of 85% (with the greatest differences at the N-terminal) and are about 80% identical to the previously described mouse orphan receptor gpr73 (Parker et al., 2000). The gene encoding for PK-R1 maps to human chromosome region 2q14 and mouse chromosome 6, while that for PK-R2 is located on human chromosome region 20p13 and mouse chromosome 2 (Kaser et al., 2003).

Data obtained from binding experiments on cell membranes exogenously transfected with PK-R1 and PK-R2 revealed a general non-selectivity of all prokineticins for either receptors, except MIT-1 and PK2Lβ that display a clear selectively for PK-R2 and PK-R1, respectively (Negri et al., 2007). The affinity of PK1 and PK2 for their receptors are in a similar range, although PK2 displays a moderately higher affinity for both receptors: K_d (nM) values for PK1 and PK2 binding to PK-R1 are 12.3 ± 4.2 and 1.4 ± 0.5, respectively, the K_d (nM) values for PK1 and PK2 binding to PK-R2 are 1.8 ± 0.1 and 2.0 ± 0.7, respectively (Lin et al., 2002a; Soga et al., 2002; Cheng et al., 2005; Maldonato-Perez et al., 2007).

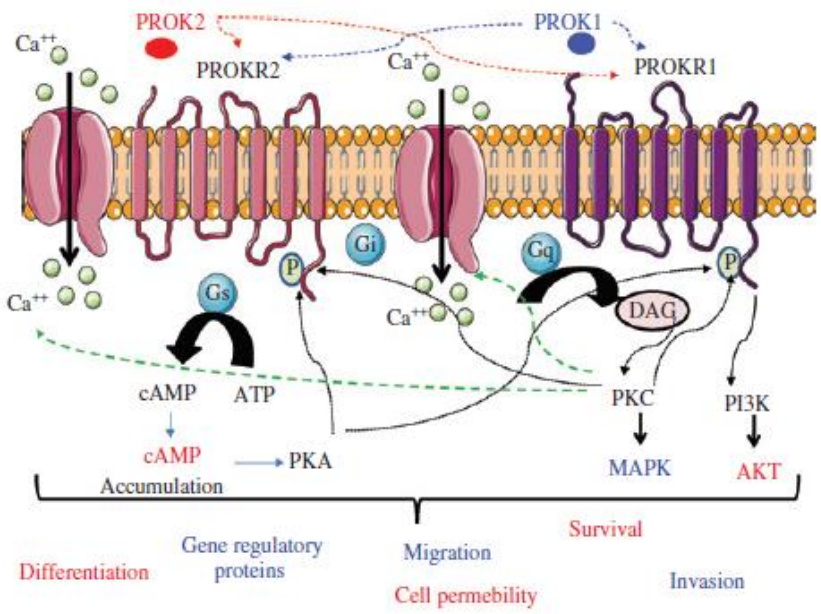


Image 12| Prokineticin signaling.
Image by Traboulsi et al., 2015.

Prokineticins can activate both of their receptor when present in nanomolar concentrations. PK-Rs activation produce a variety of downstream signalling events which ultimately result in proliferation, anti-apoptosis, differentiation and migration/mobilization of the target cells in various systems (Kaser et., 2003; Maldonado-Perez et al., 2007; Ngan and Tam, 2008). Multiple intracellular pathways are triggered by the binding of PKs to their receptors. One of the major signalling mechanisms is the intracellular calcium mobilization via protein G_q activation. PK-Rs/G_q interaction by activating phospholipase Cβ (PLCβ) promotes the formation of inositol triphosphate and the consequent calcium release from intracellular stores which leads to altered cell activity (Lin et al., 2002b).

Furthermore, intracellular calcium stimulation activates the calcineurin pathway, which induces dephosphorylation of the transcription factor, NFAT (nuclear factor of activated T cells), followed by nuclear translocation of NFAT and regulation of gene transcription (Maldonado-Perez et al., 2009; Cook et al., 2010). In the dorsal root ganglion PK/PK-Rs interaction induce an increased intracellular calcium current carried by the transient receptor potential vanilloid 1 channel (TRPV1) via a pathway involving the activation of protein kinase C (PKC) (Vellani et al., 2006). Crosstalk between the glial cell line-derived neurotrophic factor (GDNF)/Ret, TRPV1 and prokineticin signalling has also been reported (Hu et al., 2006; Ngam and Tam, 2008).

Moreover, coupling to G_i (especially PKR2) and G_s proteins, PK-Rs mediate the phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK), serine/threonine kinase Akt and cAMP accumulation, respectively (Chen et al., 2005).

PK-Rs are widely distributed in the organism and frequently, tissues with high levels of PK-R1 exhibit low to detectable levels of PK-R2 and vice versa (Martin et al., 2011).

PK-R1 is mainly, but not exclusively, present in the peripheral tissues including those of the endocrine system, gastrointestinal tract, lungs, hearth, blood cells and reproductive organs, while PKR2 is abundantly expressed in discrete nuclei of the central nervous system, particularly in the hypothalamus, the olfactory ventricular regions, the limbic system and thalamic areas (Negri et al., 2007; Cheng et., 2002; Cheng et al., 2006). PK-Rs expression has been also reported in endocrine and peripheral organs such as pituitary and thyroid glands, testis and ovary (Soga et al., 2002; Martin et al., 2011). At the level of DRG, PK-R1 and PK-R2 are expressed in different types of neurons: PK-R1, is mainly expressed on small nociceptors while PK-R2 is expressed on medium and large-sized neurons (Negri et al., 2007; Negri and Maftei 2018;). In the spinal cord, the highest density for PK-Rs is within the dorsal horns indicating that these receptors are involved in central transmission of the nociceptive signal (Negri and Lattanzi, 2012). In the same tissue, non neuronal cells, such as astrocytes and microglia mostly express PK-R1, as indicate by analysis on primary cultured neurons, astrocytes and microglia prepared from mouse cerebrum (Koyama et al., 2006).
Given their wide distribution and regulation, which vary greatly from tissue to tissue, it is easy to understand how they can display a wide range of tissue-specific biological activities. Differential G-protein expression pattern and multiple G-protein coupling of the receptors can also increase the functional complexity of the PK system (Ngam and Tam, 2008).

3.6.2 Biological functions of prokineticins

- Gastro-intestinal motility

Prokineticins were initially identified in the gastrointestinal tract as endogenous regulatory agents able to induce the contraction of isolated guinea pig ileum and promote relaxation of colon (Li et al., 2001).
The role of PKs in gastrointestinal motility was later shown also in rodents. In murine proximal colon, PK1 was found to spontaneously suppress giant contractions of the circular muscle through the release of nitric oxide (Hoogerwerf, 2006). In another study, PK2 was claimed as potential therapeutic to increase post-operative gastric and intestinal motility (Lewis, 2004).

- Neurogenesis

As previously stated PK2 and PK-R2 are widely expressed in CNS. In particular, in situ hybridization studies have detected the presence of PK2 in layer II of the cerebral cortex, in dorsal and ventral hippocampus and in cerebellar cortex of brain rodents. PK2 expression was also observed in the olfactory bulb (OB), an area where neurogenesis is present also in adulthood (Melchiorri et al., 2001).
Expression analysis of prokineticin receptors in newborn and adult rodents has shown differences in the anatomical and temporal localization of PK-Rs within the CNS depending on the rodent's age. One day after birth PK-R2 is strongly expressed in the olfactory bulb, neuroepithelium, striatum, hippocampus, thalamic and hypothalamic areas, amygdala and cortex of rat, whereas PK-R1 expression is restricted in the cortex; in the brain of adult rat only PK-R2 is still expressed. On the other hand, both receptors are highly expressed in adult DRGs (Negri et al., 2006).
PK2 was found to stimulate neuronal survival in primary cultures of cerebellar granule cells and protect cultured cortical neurons against excitotoxic death via ERK and Akt pathways activation (Melchiorri et al., 2001). Moreover, in the olfactory bulb PK2 functions as a chemoattractant inducing the migration of subventricular zone-derived neuronal progenitors and regulating OB morphogenesis (Ng et al., 2005).
Genetic studies demonstrated that PK2/PK-R2 signalling pair has a key role in the development of the olfactory bulb. PK2 and PK-R2 knockout mice display hypoplasia and defects in the structure of OB (Matsumoto et al., 2006; Negri et al., 2007). PK-R2 null mice also show severe atrophy of the reproductive system, with pathological alterations which resembles the typical clinical manifestations of Kallmann syndrome, an human developmental disease characterized by hypogonadotropic hypogonadism, due to the

lack of gonadotropin-releasing hormone neurons, and anosmia, related to defective olfactory bulb (Matsumoto et al., 2006).

- Circadian rhythm regulation

The suprachiasmatic nucleus (SCN) of the hypothalamus is the primary mammalian circadian clock that regulates daily rhythms of diverse physiological functions and behaviours, including the sleep/wake cycle, blood pressure and energy metabolism (Reppert and Weaver, 2002).

The distribution of prokineticins and their receptors in the mouse brain has revealed how PK2 and PK-R2 are abundantly expressed in SNC indicating a potential regulator function of their signalling in the circadian clock. This hypothesis was confirmed by the fact that prokineticins levels in SCN display dramatic circadian rhythmicity under light/dark conditions; hypothalamic PK2 mRNA is high during the day, while very low levels are present in the dark phase (Cheng et al., 2002). Moreover, high levels of PK-R2 are detected in the dorsal medial nucleus (DMN), and the paraventricular thalamic nuclei (PVT), brain structures also implicated in the regulation of circadian activity. Differently from the PK2 expression oscillation, PK-R2 levels show no significant diurnal alteration (Matsumoto et al., 2006).

The involvement of the PKs system in circadian rhythms regulation is also supported by studies performed using transgenic animal models. In fact, it was demonstrated that PK2-knockout mice have reduced rhythmicity for a variety of physiological and behaviour parameters, including sleep/wake cycle, activity, body temperature, circulating glucocorticoid levels, glucose levels and the expression of peripheral clock genes (Li et al., 2006). PK-R2 disruption also results in similarly damped circadian rhythms in locomotor activity and body temperature (Prosser et al., 2007).

Mood disorders are known to be linked to disrupted circadian rhythm and interestingly, in rodents, intracerebroventricular injection of PK2 results in increased anxiety-like behaviour, whereas PK2-deficient mice display reduced anxiety and depression-like behaviours (Martin et al., 2011).

- Angiogenesis and reproductive system

The angiogenic activity of prokineticins is now well accepted. PK1, also known as endocrine gland-derived vascular endothelial growth factor (EG-VEGF) for its selective angiogenic effect on endocrine glands, was found to promote angiogenesis in the ovary (LeCouter et al., 2001) and testis (LeCouter et al., 2003b) and to induce proliferation, migration and fenestration of endothelial cells derived from adrenal glands (LeCouter et al., 2001). Moreover, the presence of PK1 on mouse epithelial tubule cells and liver hepatocytes and the restricted expression of PK-R2 on endothelial cells, supported the hypothesis that PK1 also functions as a paracrine growth and survival factor for kidney and liver endothelial cells (LeCouter et al, 2003b; Ferrara et al., 2004).

Both PK1 and PK2 mediate vascular effects through the activation of their receptors, that are widely expressed in endothelial cells from different tissue vasculatures. The activation of PK-R1 enhances cell survival and proliferation while PK-R2 seems to be mostly related to the regulation of endothelial cell permeability (LeCouter et al., 2003a; Lin et al., 2002b; Kisliouk et al., 2003).

Urayama and collaborators (2007) demonstrated that PK2/PK-R1 coupling not only promotes the growth of capillary endothelia, but also modulates the cardiomyocyte survival, inducing vessel-like formation and protecting cardiac cells from oxidative stress.

PKs and PK-Rs are predominantly expressed in reproductive system in fact, testis and placenta are the sites with the highest levels of prokineticins. PK1 expression is restricted to the testosterone producing Leyding cells and believed to promote the interstitial angiogenesis to support testicular endocrine activity of testis (Maldonato-Perez et al., 2007), while PK2 is highly expressed only in the primary spermatocytes (Wechselberger et al., 1999; LeCouter et al., 2003b).

In human female reproductive organs PK2 is practically not present (Ferrara et al., 2004). On the contrary a strong expression of PK1 was found in the syncytiotrophoblast layer, the endocrine component of placenta, where PK1 could act as a novel placental growth factor for trophoblast differentiation (Maldonato-Perez et al., 2007). PK1 is also present in the ovary where it shows a dynamic pattern of expression during the ovulatory cycle and pregnancy (Ferrara et al., 2004). In addition, in the uterus PK1 was found to help implantation by increasing microvascular permeability of the endometrium (Ngam et al., 2006).

- Tumorigenesis

Prokineticins actively take part in the tumorigenesis where they act as a growth factor for cancer cells, behaving as angiogenic and a chemotactic factors for pro-inflammatory neutrophils. Aberrant PKs signalling results in hyperplasia and hypervascularity in various tissues (prostate, testicles, neuroblastoma, colon, and pancreas) and is related to the development of polycystic ovarian syndrome, neuroblastoma, colorectal, prostate and testicular cancers (Maldonato-Perez et al., 2007; Ngam and Tam, 2008). Furthermore, a potent role of PK2 released by infiltrating myeloid cells in promoting tumor angiogenesis (Shojaei et al., 2007) has been demonstrated.

Prokineticins could be implicated in other cancer types not investigated yet, such as brain cancer, because of their role as important growth factors for nerve cells of the olfactory bulb, or potentially in leukemias since they are expressed at high levels in the bone marrow by monocytic/granulocytic lineage cells.

3.6.3 Role of prokineticins in pain

As previously mentioned, PK2 is the key regulator of different biological events in the nervous system including neurogenesis, circadian rhythms and pain processing.

In particular, PK2 enhances pain sensitivity through the activation of PK-R1 and PK-R2, widely expressed in nervous tissues related to pain transmission, such as dorsal root ganglion (DRG), outer layers of the dorsal horns of spinal cord and in the peripheral terminals of nociceptor axons, as well as in cells participating to immune and inflammatory responses (Negri et al., 2006; Negri and Lattanzi, 2012).

The involvement of the PKs system in nociception was suggested by the observations that intraplantar injections of low doses of PK2 in rodents induced a strong and localized hyperalgesia development as demonstrated by the decrease in nociceptive thresholds to thermal and mechanical stimuli (Mollay et al., 1999; Negri et al., 2002).

It was also demonstrated that PK2 produced a biphasic hyperalgesia to tactile and thermal stimuli when administered in rats by systemic routes. The first hyperalgesic peak was caused by a local action of PK2 on nociceptors, because when PK2 was injected intraplantar, it induced hyperalgesia with a similar time course to that of the initial phase of hyperalgesia seen with systemic injection, while the second phase of hyperalgesia was probably related to a central action of PK2 as it is not present after the intraplantar injections. (Negri et al., 2002; Vellani et al., 2006).

PK2 was found to promote central sensitization enhancing the release of mediators implicated in pain processing as Calcitonin-Gene Related Peptide (CGRP) and substance P (SP). PK2-responding neurones contain and release CGRP and SP upon exposure to PK2. Moreover, i.t. injection of PK2 in rats results in an up-regulation of CGRP and SP immunoreactivity in lumbar dorsal horn and in DRG (DeFelice et al, 2012).

PK-R1 and PK-R2 are highly expressed in DRG neurons. In vitro culture of rats DRG neurons showed that PK2 significantly lowers the nociceptive thresholds to a broad spectrum of physical and chemical stimuli and subsequent studies showed the ability of PK2 to induce nociceptors sensitization through the modulation of TRPV1, channel notoriously involved in pain transmission (Negri et al., 2006; Vellani et al., 2006). Functional assay revealed in fact that PK2 dose-dependently mobilize intracellular calcium

promoting the translocation of PKCε from cytoplasm to neuronal membrane, which activation is responsible for the phosphorylation and the consequent sensitization of TRPV1 (Numazaki et al., 2002).
These functional studies are consistent with results of colocalization experiments indicating that the majority of PK-R1-positive DRG neurons also express TRPV1 (Hu et al., 2006). The critical role of TRPV1 in the downstream signalling of PK-R1 in pain perception is further supported by the reduced response of TRPV1 deficient mice to PK2 (Negri et al., 2006; Zhou et al., 2006).
A possible involvement of PK system in central modulation of pain has been demonstrated by DeNovellis and collaborators. Their study reports that intra-periaqueductal grey (PAG) administration of PK2 induced a pro-nociceptive action increasing the intrinsic GABAergic tone which, in turn, was responsible for the inhibition of PAG anti-nociceptive output neurons impinging on RVM neurons (DeNovellis et al., 2007).
A crucial role for PK2 in mediating inflammatory hyperalgesia was documented by Giannini et al (2009). They showed that in a mouse model of inflammatory pain induced by complete Freund's adjuvant (CFA), a strong PK2 increase was detectable in granulocytes and macrophages and, furthermore, this increase was correlated not only to the development but also to the duration of pain.
Prokineticin system contribution to pain sensitivity was demonstrated also in several murine models of neuropathic pain. Our laboratory previously demonstrated how in diabetic mice there was a correlation between PK2 levels in the nervous tissues related to pain transmission and the duration and degree of the painful symptomatology (Castelli et al., 2016).
The same correlation was observed in the spared nerve injury (Guida et al., 2015) and in the chronic constriction injury model (Maftei et al., 2014). Furthermore, in these studies it was shown how in peripheral nervous system (sciatic nerve and DRG) PK2 and PK-R1 was mainly associated to activated macrophages, while in the central nervous system (spinal cord) the main producers of PK2 seemed to be proliferating and activated astrocytes.
Evidence for a direct involvement of PKs/PK-Rs pair signalling in modulating pain processing was also supported by studies of PK2-, PK-R1- or PK-R2- knockout mice (Negri et al., 2006; Hu et al., 2006). Animals lacking the PK-Rs or PK2 exhibit impaired pain perception to various stimuli, including noxious heat, mechanical stimuli and capsaicin. PK-R1-deficient mice also display an impaired development of hyperalgesia after tissue injury.

3.6.4 Function of Prokineticin in immune system

In human and mouse, hematopoietic stem cells, lymphoid organs, and peripheral blood cells (peripheral leucocytes, neutrophils, dendritic cells and monocytes) as well as resident organs immune cells constitutively express moderate levels of prokineticins and their receptors (LeCouter et al., 2004; Dorsch et al., 2005).
By coupling to its receptors, prokineticin 2 modulates growth, survival and cell function of both innate and adaptive immune system. The involvement of the PKs system in haematopoiesis and in regulatory processes associated to inflammatory and immune responses is reported in numerous studies (LeCouter et al., 2004).
It was demonstrated that both PK1 and PK2 support the differentiation of mouse and human bone marrow cells into monocyte/macrophage lineage (Dorsch et al., 2005; LeCouter et al., 2004).
Moreover, prokineticins were shown to regulate the functioning of several immune cells. PK1 and PK2 are in fact highly expressed on different mature blood cells types. PK2 expression is particularly associated to monocytes, neutrophils and dendritic cells, and this protein is extensively released by neutrophils at the site of inflammation, where it stimulates the migration of monocytes (LeCouter et al., 2004).
PK1 promotes monocytes activation inducing changes in cell morphology and expression levels of cytokines or cytokines receptors (Dorsch et al., 2005), while PK2 was found to modulate murine macrophage and

spleen lymphocyte activity affecting both innate and acquired immunity functioning (Martucci et al., 2006; Franchi et al., 2008). In particular, our group demonstrated PK2 ability to induce macrophage migration and acquisition of a pro-inflammatory phenotype which lead to a significant increase in LPS-induced production of the pro-inflammatory cytokines IL-1β and IL-12, and a decrease of the anti-inflammatory cytokine IL-10 (Martucci et al. 2006). PK2 also influences T lymphocyte population skewing the Th1/Th2 balance towards a Th1 response (Franchi et al., 2008). All these effects are mediate by the activation of PK-R1, in fact they cannot be detected in PK-R1 knockout mice, revealing a pivotal role of this receptor in the regulation of immune response (Martucci et al., 2006; Franchi et al., 2008).
Very little is known about the molecular mechanisms of regulation of prokineticin and prokineticin receptors within immune cells (Monnier and Samson, 2008). However, PK2 transcription was recently demonstrated to be positively regulated in CD11b+Gr1+ bone marrow-derived cells (i.e. neutrophils and cells of the macrophage lineage), specifically by granulocyte colony-stimulating factor (G-CSF) (Shojaei et al., 2007).
It has also been shown that human monocytes exposed to PK2 induced extracellular signal-regulated kinase phosphorylation that was abolished by pertussis toxin, suggesting involvement of the G_i protein signaling pathway (LeCouter et al., 2004). Interestingly, in mouse macrophages, it seems that pertussis toxin was not able to block PK2, but rather inhibition of the G_q protein pathway stopped the PK2-mediated secretion of cytokines mediated by PK2 (Martucci et al., 2006).
Prokineticins were also found to be involved in inflammatory diseases and cancer. Expression analysis revealed high levels of PK1 in rheumatoid arthritis synoviocytes (Dorsch et al., 2005) and PK1 transcripts were also detected in tumor-infiltrating T lymphocytes in ovarian carcinoma (Kisliouk et al., 2007).
PK2 is particularly overexpressed in inflamed appendix and tonsil where it is associated to infiltrating immune cells. It was also clearly demonstrated a role of PK2 in mediating inflammatory hyperalgesia induced by CFA injection (Giannini et al., 2009). From this study also results a clear involvement of both PK-Rs in inflammatory pain as in mice lacking PK-R1 or PK-R2 a significant less inflammation-induced hyperalgesia was observed in comparison with WT mice. However, the inflammation-induced PK2 upregulation that was significantly reduced in PK-R1 knockout mice than in WT and PK-R2 null mice, once again demonstrating a specific involvement of PK-R1 in setting the enhanced PK2 levels during inflammation (Giannini et al., 2009; Negri and Lattanzi, 2011).
It was supposed that the activation of PK-R1 on granulocytes and macrophages by PK2 released at the site of inflammation could promote their recruitment (Martucci et al., 2006) and survival by paracrine and autocrine mechanisms, directly or synergizing with G-CSF (Shojaei et al., 2007; LeCouter et al., 2004).

3.6.5 Prokineticin receptors antagonists

Considering the involvement of the PKs system as peripheral and central mediators of a wide range of biological functions and pathological conditions in various tissues, the development of effective PK-Rs antagonists may be useful in the treatment of various diseases.
Since PK2 was found to be a potent pro-nociceptive/pro-inflammatory agent, regulating pain processing, it follows that antagonism of PKs signalling could represent a promising approach to handle different kinds of pain.
The identification of the structural determinants necessary for the binding to the receptors and activity of PKs is mandatory for the design of effective PK-Rs antagonists (Balboni et al., 2008). As previously described, the amino terminal sequence AVITGA and the tryptophan (Trp) residue in position 24 are essential for PKs/PK-Rs binding and their biological activity.
Miele and collegues suggested that AVIT proteins could interact with PK-Rs by orienting the protein region including the AVITGA sequence and the conserved Trp24 (Miele et al., 2010). Moreover, it was

demonstrated that deletions and substitutions in these conserved residues produce antagonist molecules (Bullock et al., 2004; Negri et al., 2005).

The N-terminal deletion of the first two amino acids (alanine and valine) in Bv8 molecule (dAV-Bv8), yields an analogue lacking any biological activity but still able to bind the receptors acting as PK-Rs antagonist both in vitro and in vivo (Negri et al., 2005). The substitution of Trp with Ala in position 24 produces another antagonist-like protein, A-24, which, preferentially binding PK-R2, was found to exert a long-lasting anti-hyperalgesic effect in animal models of postsurgical and inflammatory pain (Lattanzi et al., 2012). However, the big size of these peptides makes difficult and expensive their use for clinical therapy.

Recently, new promising non-peptidic PK-Rs antagonists, triazine-guanidine derivates, have been synthetized and developed. The "lead compound" of this class is a molecule named PC1. PC1 mimics the structural features required for PK-Rs binding; the triazine-guanidine moiety of the molecule mimics the N-terminal AVIT sequence, whereas the methoxybenzyl moiety is oriented as the tryptophan residue in position 24 (Balboni et al., 2008).

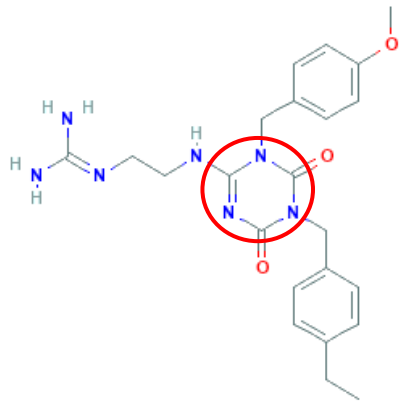


Figure 13 | PC1 chemical structure.
Red circle indicates triazine group.
Image edited by PubChem.

Results from binding assay demonstrated that PC1 is a PK-R1-preferring ligand. This molecule displays in fact an affinity about 70-fold higher for PK-R1 (Ki=22 nM) than for PK-R2 (Ki=1610 nM).

In vitro studies revealed a clear antagonist activity of PC1 as it was found to inhibit Bv8-induced intracellular calcium mobilization in PK-R1- and PK-R2-transfected CHO cells (Balboni et al., 2008). Furthermore, in vivo studies demonstrated that PC1 is able to selectively antagonize Bv8-induced hyperalgesia and capsaicin-induced thermal hypersensitivity, suggesting that it may prevent activation of PK-Rs and TRPV1 by their endogenous ligands (Negri and Lattanzi, 2012).

In CFA-induced inflammatory pain model, systemic injections of PC1 (from 20 to 150 µg/kg, s.c.) reduced hyperalgesia in dose-dependent manner, completely abolishing it at the dose of 150 µg/kg (Giannini et al., 2009). The same dose-dependent effect was observed also in a model of neuropathic pain (Maftei et al., 2014), where a single bolus systemic injection of PC1 (30, 75 and 150 µg/Kg) was able to reduced both thermal hyperalgesia and tactile allodynia.

Furthermore, in murine models of spared nerve injury (Guida et al., 2015) and painful diabetic neuropathy (Castelli et al., 2016), chronic treatment with PC1 (150 µg/kg, s.c.) was able to decrease hypersensitivity in response to thermal and mechanical stimuli. Interestingly, in the diabetes model, the PK-Rs antagonist was able to exert a preventive effect when administered at the same time of diabetes induction (Castelli et al., 2016).

4. Aim of the research project

Chemotherapy-induced peripheral neuropathy is a common and challenging complication due to treatment with many commonly used anticancer agents. This neuropathic pain syndrome interferes with optimal treatment of disease resulting in the need for dose reduction, treatment delays and even premature cessation of chemotherapy, and can lead to long-term debilitating effects that can cause increased morbidity and decreased quality of life (Brewer et al., 2016). The main sensory symptoms commonly reported include allodynia, hyperalgesia, paresthesia, dysesthesia or pain that is burning, shooting or electric-shock-like (Colvin et al., 2019). CIPN has a typical "stocking and glove" distribution but then the pain can expand all over the body. Although in many patients, acute CIPN will resolve after the end of the chemotherapeutic schedule, in a number of cases, it will persist, resulting in chronic symptoms, months or even years later. In some cases, CIPN can appear shortly after finishing the chemotherapeutic treatment, a phenomenon called "coasting". Therefore, patients may well be cancer-free, but suffering a debilitating neuropathy evoked by their cancer treatment. Up to now there are no valid treatments to handle this neuropathy, so there is an urgent need of novel therapeutic approaches. The mechanisms at the basis of CIPN are complex, with peripheral, spinal and supraspinal changes such as alterations in ion channels, mitochondrial dysfunction and, as it happens also in other forms of neuropathic pain, there is a strong involvement of neuroinflammation.

In the last years it has been recognized that cytokines and chemokines are potent neuromodulators involved in neuroinflammation at different anatomical locations, including peripheral injured nerve, DRGs, spinal cord and brain and contribute to chronic pain processing (Abbadie et al., 2009; Gao and Ji, 2010). In the pathological and complex network between neuronal and non-neuronal cells along the way of pain transmission, increasing evidences now suggest also a possible implication of a the Prokineticins (PKs),a family of chemokine-like proteins. Prokineticin 2 (PK2) belongs to this family of chemokines and was found to have a pivotal role in pain transmission and immunomodulation acting on its receptors PK-R1 and PK-R2 widely distributed in the central nervous system, DRG neurons and in cells participating to immune and inflammatory response (Negri et al., 2007). PK2 is especially active in lowering pain thresholds and displays a major role in triggering inflammatory pain (Negri et al., 2006; Giannini et al., 2009); it is involved in neuronal sensitization through co-operative interaction with TRPV1 channels in DRGs and promotes the release of neuromediators implicated in pain processing as CGRP and substance P (DeFelice et al., 2012). In addition, PK2 has a role in regulating the immune response. In fact, the stimulation of PK-R1 induces a pro-inflammatory phenotype in macrophages, activating these cells to migrate and produce pro-inflammatory cytokines; PK2 also induces the shift towards a Th1 profile in lymphocytes (Martucci et al., 2006; Franchi et al., 2008). Considering that PK-R1 and PK-R2 as well as PK2 are expressed in neurons, glia and immune cells and that this system is involved in nociception and immunoregulation, PK2/PK-Rs pair might exerts a critical role in chronic pain transmission (Koyama et al., 2006).

On these premises, the aim of the present study was to investigate the role of the Prokineticin system in the onset and chronicization of chemotherapy induced peripheral neuropathy by using experimental mouse models characterized by the administration of two different chemotherapeutic agents commonly used in oncology and related to CIPN development: Vincristine (VCR), the most neurotoxic drug among Vinca Alkaloids, and Bortezomib (BTZ), the first of a new class of agents that inhibit the 26s complex of proteasome favouring apoptosis of cancer cells. Hypersensitivity was evaluated by measuring mechanical and thermal allodynia as well as thermal hyperalgesia, which are the most frequent symptoms referred by CIPN patients. In order to understand if PK2, PK-R1 and PK-R2 activities were necessary for the onset, maintenance or resolution of neuropathic pain, mice were chronically treated with the PK-Rs antagonist PC1, which was proved to be effective in inflammatory (Giannini et al., 2009) and neuropathic (Castelli et al., 2016; Guida et al., 2015; Maftei et al., 2014) pain treatment.

Different approaches and complementary methods (behavioural, biochemical and hystochemical analysis) were used to prove the existence of a correlation between the presence of hypersensitivity and the activation of the prokineticin system.

In order to identify the mechanisms responsible for the effect of the PK-Rs antagonist PC1 we evaluated expression and protein levels of PK system components before starting PC1 chronic treatment and at the end of the PC1/chemotherapeutic schedule in the main stations involved in nociception transmission. In the same tissues, neuroinflammation was assessed by measuring pro- (TNF-α, IL-6, IL-1β) and anti-inflammatory (IL-10) cytokine expression levels. In order to better investigate the immune/inflammatory components, we studied the macrophage/Toll Like Receptor 4 (TLR4) pathways. In fact, it is known that the recruitment and infiltration of macrophages in peripheral nerves and especially in the DRG has a key role in the development of CIPN and since PK2 is able to induce the macrophage to migrate and to acquire a pro-inflammatory phenotype, we decided to evaluate, in the peripheral (DRG and sciatic nerve) and central (spinal cord) nervous system, expression levels of CD68 and CD11b which are routinely used as markers of macrophages and of TLR4, which activation is linked to pro-inflammatory cytokines modulation.

It is known from previous studies that in the central nervous system PK2 is expressed by both microglia and astrocytes, in order to understand the possible involvement of these two cell types, both expression levels and immunostaining of CD68 and GFAP were evaluated at the level of spinal cord.

Furthermore, in order to better understand the relationship between these cells and PK2, in the BTZ model, we also performed an immunostaining to colocalize PK2 with CD68 and GFAP. To assess a possible cell stress/neuronal injury, we tested mRNA levels of Activating Transcription Factor (ATF3), an adaptive response gene whose activity is usually regulated by stressful stimuli.

In order to more closely resemble the clinical situation, where patients are often subjected to several cycle of chemotherapy (Starobova and Vetter, 2017), we monitored mechanical allodynia after the interruption of the chemotherapy/PC1. Once mice were fully recovered they were subjected to a second chemotherapeutic/ PC1 treatment to assess whether the PK-Rs antagonist could exert a protective effect in hypersensitivity development during a second VCR/BTZ cycle.

Given the widely known susceptibility of DRG to toxic chemotherapeutic agents (Montague and Malcangio, 2017), in the second part of our study we decided to investigate their action on the DRG neuronal, by studying the effect of BTZ and VCR on primary sensory neurons. In particular, we focused on neurites outgrowth, an important morphological phenotype of neuronal cells that correlates with their function and health (da Silva et al., 2002).

The effect of different concentrations of BTZ and VCR on neurite outgrowth of DRG primary sensory neurons were tested (Alè et al., 2015) alone or in association to different concentrations of PC1 (Severini et al., 2015).

Expression levels of PK system, pro- and anti- inflammatory cytokines and markers of other cell types (satellite cells) possibly present in the cell cultures were analyzed. Immunofluorescence was used in order to study the localization of PK2 on neuronal cells.

These results can help us to understand if the Prokineticin system is involved in CIPN caused by BTZ and VCR, and whether PK-Rs antagonism, through the administration of PC1, is effective in contrasting the development and maintenance of hypersensitivity associated to chemotherapeutic treatment.

5. Materials and Methods

5.1 Animals

Male nine-weeks-old C57BL/6J mice (Charles River Laboratories, Calco, Italy) were used in all the experiments. Animals were housed in groups of three in translucent polyethylene cages. They were housed with light/dark cycles of 12 hours, temperature of 22 ± 2 °C, humidity of 55 ± 10%, standard rodent chow and tap water *ad libitum*. Animals were acclimatized to the environment for 7 days before starting the experiments.

All the procedures performed on animals were in compliance with international policies (EEC council directive 86/609, OJ L 358, 1 Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, Us National Research Council, 8 th ed., 2011) and were approved by the Animal Care and Use Committee of the Italian Ministry of Health (permission number 709/ 2016 to Silvia Franchi). All efforts were made to reduce the number of animals used and to minimize animal suffering in accordance with 3R principles.

5.2 Induction of painful neuropathy

Chemotherapy-induced peripheral neuropathy was induced in mice through the administration of two different antineoplastic agents: bortezomib or vincristine. The chemotherapeutic protocol for both drugs was assumed from the literature (Boehmerle et al., 2014; Kiguchi et al., 2008). The schedule treatments allow to induce a detectable painful neuropathy minimizing unspecific systemic toxicity and preventing mice death.

5.2.1 Bortezomib

Bortezomib, BTZ, (LC Laboratories, Woburn, USA) was freshly prepared before each administration. BTZ was dissolved in dimethysulfoxide 10% (DMSO) with a concentration of 1 mg/ml and diluted in sterile 0,9 % NaCl (saline) solution to final concentration of 40 µg/ml (Boehmerle et al., 2014) and it was intraperitoneally (i.p.) injected at the dose of 0,4 mg/kg.
For each cycle, BTZ or vehicle was administrated 3 times a week for 4 consecutive weeks (cumulative injected dose: 4,8mg/kg). Control mice were i.p. injected with vehicle.

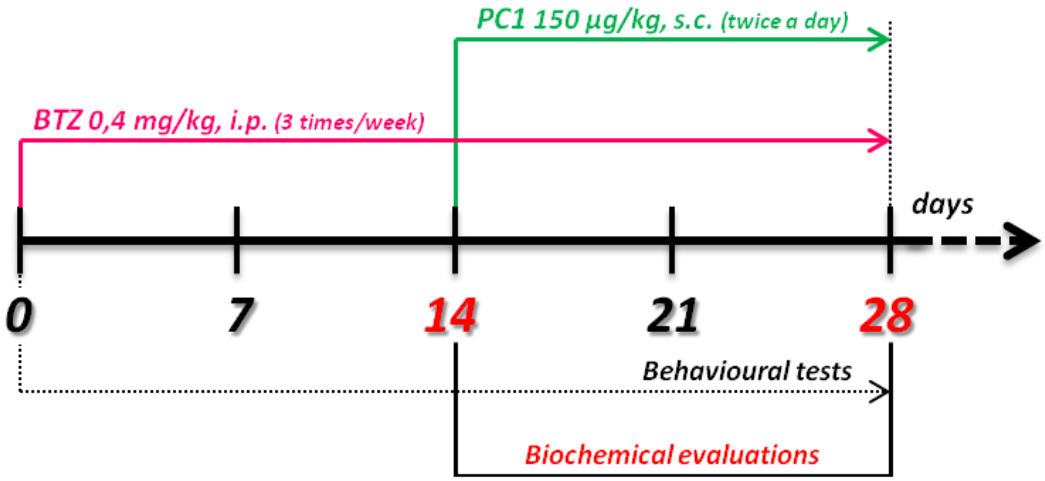


Image 14 | BTZ schedule.

5.2.2 Vincristine

Vincristine sulfate, VCR, (TOCRIS Bioscience™, Minneapolis, USA) was dissolved in water for injections with a concentration of 1 mg/ml, diluted in sterile saline solution (0,9% NaCl) to final concentration of 10 µg/ml and it was intraperitoneally (i.p.) injected at the dose of 0,1 mg/kg (Kiguchi et al., 2008).
For each VCR cycle, the chemotherapeutic agent was daily administered for 14 consecutive days (cumulative injected dose: 1,4 mg/kg). VCR was freshly prepared before each administration. Control mice were i.p. injected with vehicle (saline solution).

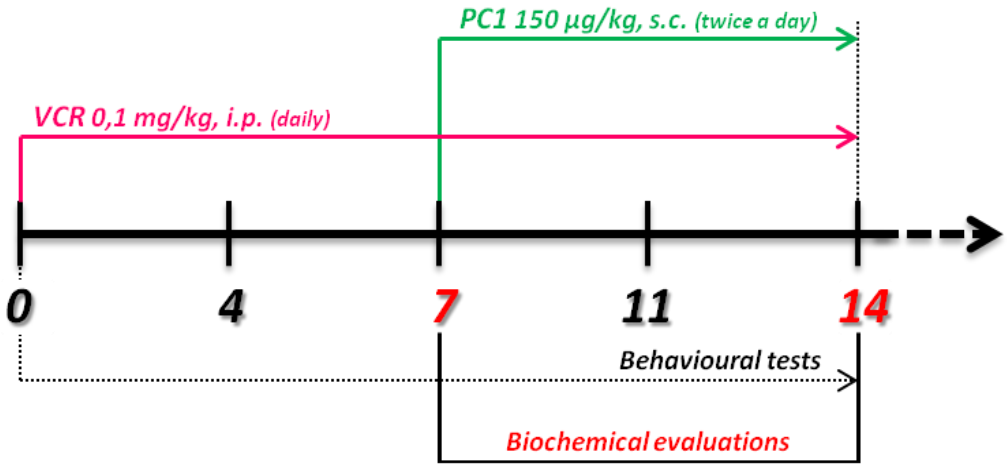


Image 15| VCR schedule.

After a classical single BTZ (28 days) or VCR (14 days) cycle, mice were monitored over time for the presence of mechanical allodynia, until they completely recovered to control values. At this time point (i.e. 84 and 80 days after the first BTZ- or VCR- injection, respectively), mice previously treated with the antineoplastic drug, started a second BTZ or VCR cycle (using the before mentioned protocols). Control mice were i.p. injected with vehicle (saline solution).

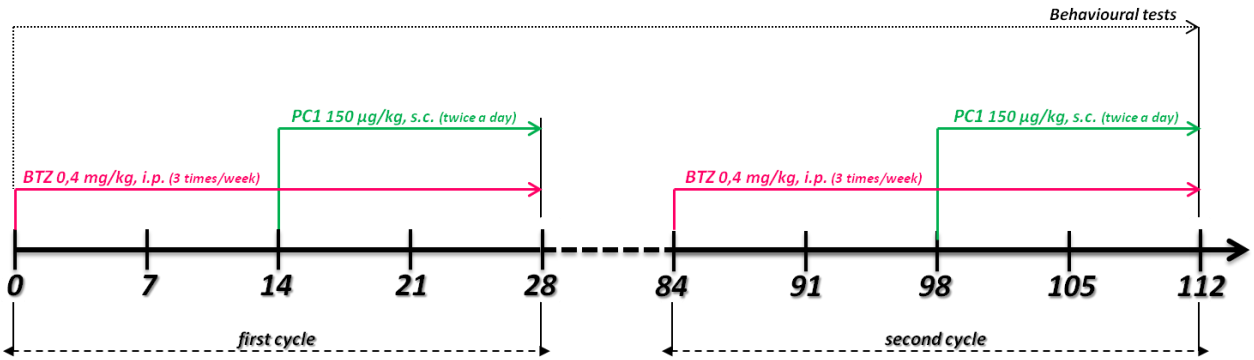


Image 16| BTZ multiple cycles.

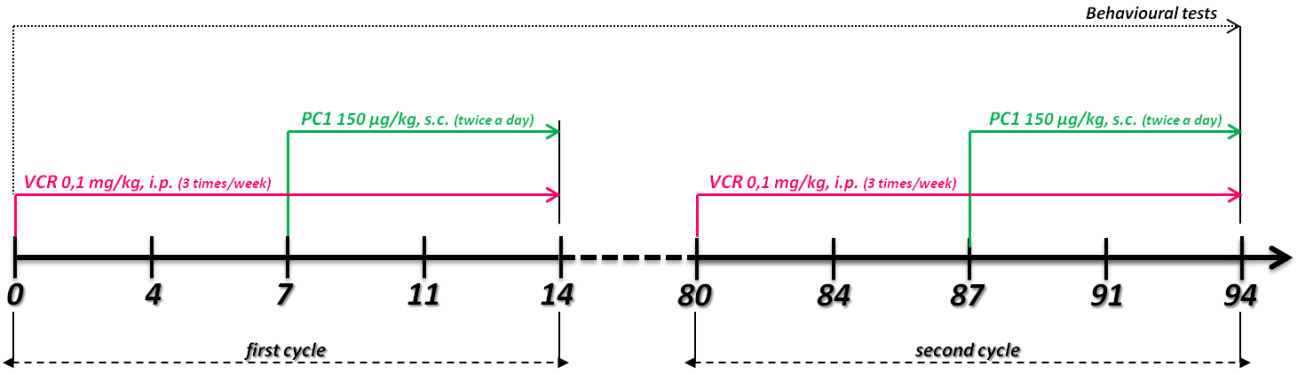


Image 17| VCR multiple cycles.

5.3 PC1 therapeutic treatment

The PK-Rs antagonist PC1, a triazine-guanidine compound (Balboni et al., 2008), was prepared by weighing PC1 powder and dissolving it in a DMSO 10% - sterile deionized water solution. This stock solution was then diluted to the final concentration in sterile saline solution. Mice were treated with PC1 150 µg/kg, subcutaneously (s.c.) injected twice a day for several consecutive days, depending on the chemotherapeutic drug schedule. In particular, mice subjected to a single BTZ cycle were treated with PC1 or saline for 14 consecutive days starting from the 14th day after the first BTZ administration, a time point corresponding to a well established hypersensitivity development.

VCR-treated animals received PC1 or saline administrations for 7 consecutive days starting 7 days after the first VCR injection, when they were already in an evident state of hypersensitivity.

PC1 dose was choosen on the basis of previous experiments, where 3 doses (30, 75 and 150 µg/kg) of PC1 were tested on chronic constriction injury (CCI)-induced thermal hyperalgesia and mechanical allodynia. The maximum effect of PC1 was achieved at the highest dose of 150 µg/kg. Based on these results, the dose of 150 µg/ kg of PC1 was chosen for chronic treatment not only in CCI model (Maftei et al., 2014) but also in other models of neuropathic pain such as SNI (spared nerve injury) (Guida et al., 2015) and streptozotocin model of diabetic neuropathy (Castelli et al., 2016). Control animals were injected with an equal amount of sterile saline solution.

Mice treated with PC1 during the first chemotherapeutic cycle and then subjected to the second BTZ or VCR schedule were treated again with the PK-Rs antagonist (using the same experimental schedule of the first cycle).

5.4 Characterization of nociceptive behaviour

Chemotherapy induced peripheral neuropathy development was evaluated by measuring the most frequent symptoms encountered in neuropathic patients, i.e. mechanical and cold allodynia as well as thermal hyperalgesia, monitoring over time the responses of the animals to thermal and mechanical stimuli.

All behavioural tests were carried out in the morning in a quiet temperature-controlled room after a habituation period of 30 minutes. All behavioural evaluations were always performed by researchers who were blind to treatments. For all the behavioural tests the response to the different stimuli was measured three times on both hind paws and the mean of the values was then calculated.

Responses to mechanical and thermal stimuli were assessed in mice before starting chemotherapeutic protocol (day 0) and:

- 7, 14, 21 (corresponding to 1 week of PC1 chronic treatment) and 28 (corresponding to 2 weeks of PC1 chronic treatment) days after the 1st BTZ injection;
- 4, 7, 11 (corresponding to 4 days of PC1 chronic treatment) and 14 (corresponding to 1 week of PC1 chronic treatment) days after the 1st VCR injection.

In order to closely resemble the clinical situation, where patients are subjected to multiple cycle of chemotherapy, we investigated the effect of a second BTZ or VCR cycle and the action of another chronic treatment with the antagonist PC1 on mechanical allodynia after the interruption of the 1st BTZ and VCR and during a second chemotherapeutic schedule. Therefore, in mice subjected to multiple chemotherapy schedule behavioural analysis were also performed at day:

- 30, 49, 56, 63, 77, 84, 91, 98, 105 and 112 after the 1st BTZ injection;
- 21, 65, 76, 80, 84, 87, 91 and 94 after the 1st VCR injection.

Considering the time span of the acute effect of PC1 (Castelli et al., 2016), to test the effect of the chronic treatment with PC1, all behavioral tests were performed at least 12 hours after the last injection of the antagonist.

Our laboratory previously showed (Castelli et al., 2016) that a single bolus of PC1 in neuropathic mice (streptozotocin model, diabetic polyneuropathy) was able to promote a total recovery of paw withdrawal threshold (PWT) within 30 minutes. The anti-allodynic effect lasted about 4 hours, with a maximal efficacy 2hours after its injection, then it progressively disappeared.

The length of the acute benefic effect of a single bolus of PC1 was studied both in BTZ- and VCR-injected mice at the end of the chemotherapeutic protocol (BTZ day 28 and VCR day 14). Mechanical allodynia was assessed 30, 60, 120 and 240 minutes after PC1 injection.

5.4.1 Von Frey test: mechanical allodynia evaluation

Mechanical allodynia, a painful response to innocuous mechanical stimuli, was monitored evaluating the mechanical touch sensitivity through a blunt probe (Von Frey filament, 0.5 mm diameter) on the mid plantar surface of the animal hind paw, using the Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy).

This instrument consists of a moveable force actuator containing the Von Frey filament, which is placed below a metallic mesh platform upon which the researchers position the animal, whose mobility is restrained by a Perspex cage. The moveable force actuator is a cylindrical vessel equipped with an adjustable angled-mirror in order to place the touch probe below the target area of the paw and, start keys that when pressed start the vertical movement of the Von Frey filament. A controller allows the setting of the force exerted by the filament on the mouse paw and the reading (in grams) of the paw withdrawal threshold (PWT). From the practical point of view, an increasing force (ranging up to 10 grams in 10 seconds) starting below the threshold detection is applied on the mid plantar surface of the hind paw; when animals feel pain they remove their paw and the PWT is recorded.

5.4.2 Cold allodynia: thermal allodynia evaluation

Cold allodynia was measured by using Acetone drop test as previously described by Flatters and Bennett (2004). A drop (50µl) of acetone was gently applied on the mid-plantar surface of the hindpaw and the mouse was immediately placed in perspex box (size: 60cm × 40 cm × 15 cm); a stopwatch was started. The response of the animal to the cooling effect of acetone was monitored for 40 s and graded to a 4-point scale. If the mouse did not withdraw, flick or stamp its paw within this 40 s period then no response was

recorded (0, see below). However, if within the 40 s period the animal responded to the cooling effect of the acetone, then the animal's response were graded to the following 4-point scale:
- ***0***, no response;
- ***1***, quick withdrawal, flick or stamp of the paw;
- ***2***, prolonged withdrawal or repeated flicking (≥2) of the paw;
- ***3***, repeated flicking of the paw with licking directed at the ventral side of the paw.

5.4.3 Plantar test: thermal hyperalgesia evaluation

Thermal hyperalgesia, an increased pain sensitivity to thermal painful stimuli, was assessed according to the Hargreave's procedure, using the Plantar Test Apparatus (Ugo Basile, Comerio, Italy).
The instrument basically consists of a movable cylindrical vessel of aluminium which contains the infrared (I.R.) generator, which is placed below a glass panel upon which the operator put the animals. Three compartments, further subdivided by plexiglass structures (w11xh11cm), in order to test at the same time more animals, divide the Perspex box and delimit the space within which the animals are free to move.
The instrument is equipped with a controller that allows the setting of the I.R. rays intensity (100 mW/cm^2 for mouse) and the reading (in seconds) of the paw withdrawal latency (PWL) time.
Briefly, the operator places the movable I.R. generator under the glass panel at the level of the mid-plantar surface of the hind paw of the animal and by pressing the start key the heat stimulation begins. When mice feel pain and remove their paw, the I.R. source switches off and the reaction time, i.e. PWL, is recorded. In our experiments, the cut-off time was set at 22 seconds in order to prevent tissue damage.

5.5 Sacrifice and tissue sampling: spinal cord, dorsal root ganglia and sciatic nerve collection

Before starting PC1 chronic treatment (BTZ day 14 and VCR day 7) and the end of the chemotherapeutic/PC1 schedule (BTZ day 28 and VCR day 14), mice were euthanized by CO_2 inhalation.
Spinal cord (L4-L6), DRG (L4-L6) and right and left sciatic nerve (L4-L6) were dissected from neuropathic mice treated with either saline or PC1 and their respective controls, flash-frozen in liquid nitrogen and stored at -80°C until further processing.

5.5.1 RNA extraction and RT-qPCR

Total RNA was isolated from sciatic nerves (L4-L6), DRG (L4-L6) and lumbar (L4-L6) spinal cord using TRIzol® reagent (Invitrogen, ThermoFisher Scientific, Italy) according to manufacturer's instructions and re-suspended, depending on the tissue, in 10–20 µl of RNase-free water.
Before proceeding to RNA quantification, RNA samples underwent to DNAse treatment (DNA-free™ DNase kit Treatment and Removal Reagents, Ambion, Applied Biosystem, Italy) to avoid false-positive results due to contaminating DNA genomic amplification. RNA quantity and quality was determined using a BioPhotometer (Eppendorf, Germany). From each sample an equal amount of RNA (1000 ng) underwent to reverse-transcription using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Italy) and it was used as template for the Reverse transcription quantitative polymerase chain reaction, RT-qPCR.
Gene of interest were analyzed by Reverse transcription quantitative polymerase chain reaction (RT-qPCR) using the following TaqMan Gene Expression Assays (Table 2) (Thermofisher Scientific, Waltham, USA).
RT-qPCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystem, Foster City, CA) and carried out in a final volume of 20µl consisting of: 2µl (corresponding to 100ng of cDNA), 10µl of Luna®Universal Probe qPCR Master Mix (New England BioLabs, Ipswich, MA), 7µl of RNAse free water and 1µl of the following TaqMan Gene Expression Assays (Thermofisher Scientific):

Gene	code
Prokineticin2 (PK2)	Mm00450080_m1
PK-R1 (Prokr1)	Mm00517546_m1
PK-R2 (Prokr2)	Mm00769571_m1
IL-1β	Mm00434228_m1
TNF-α	Mm00443258_m1
IL-6	Mm00446190_m1
IL-10	Mm00439616_m1
CD68	Mm03047343_m1
CD11b	Mm00434455_m1
TLR4	Mm00445274_m1
GFAP	Mm01253033_m1
ATF3	Mm00476033_m1
GAPDH	Mm99999915_g1

Table 2 | TaqMan Gene Expression Assays.

Experimental procedures were performed according to the TaqMan Gene Expression Assays protocol. Each sample was run in duplicates alongside non-template controls. The PCR cycle protocol used was: 1 min at 95°C, 40 five-step cycles of 15 s at 95°C and 30sec at 60°C. Threshold cycle numbers (Ct) of the specific gene of interest and the endogenous control gene GAPDH were determined by ABI PRISM 7000 Sequence Detection System (AppliedBiosystems®, Foster City, USA). The Ct value of the specific gene of interest was normalized to the Ct value of the endogenous control, GAPDH, and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was then applied using the specific control group (vehicle treated mice) as calibrator.

5.5.2 Fluorescent immunohistochemistry

5.5.2.1 Nervous tissues collection for immunohistochemical analysis

At the end of BTZ/VCR/PC1 treatments, anesthetized mice were transcardially perfused with 30 mL of saline phosphate buffer (PBS 1X, pH 7.4), followed by 60 mL of cold 4% paraformaldehyde. L4-L6 spinal cord, DRG, and sciatic nerve were dissected from transcardially perfused mice (PBS followed by 4% paraformaldehyde (PFA)), post-fixed in 4% PFA for 24 h, cryoprotected in 30% sucrose solution, embedded in cryostat medium, and frozen and cut using a cryostat.

Prior to immunofluorescence staining, all sections were blocked with 3% normal donkey serum, containing 0.1% Triton X-100 for 30 min at room temperature. Spinal cord transverse sections (40 µm, free-floating) were incubated at 4 °C for 48 h, whereas DRG and sciatic nerve sections (20 µm, mounted on slides) were incubated at 4 ° C overnight with the following primary antibodies diluted in PBS-0.3% Triton X-100: anti-PK2 (rabbit, 1:200, AbCam, Cambridge, UK), anti-PK-R1 and anti-PK-R2 (rabbit, 1:200, Alomone labs, Jerusalem, Israel), anti-GFAP (mouse, 1:400, Immunological Sciences, Italy), and anti- CD68 (mouse, 1:400, AbCam, Cambridge, UK). After washing, sections were incubated for 2 h at room temperature with anti-species IgG secondary antibodies coupled to Alexa Fluor®-488 or 555 (1:200, Immunological Sciences). Nuclei were stained with DAPI (1:500, Sigma Aldrich). Possible non-specific labeling of secondary antibodies was detected using secondary antibody alone. Images of stained sections were acquired using a laser-scanning confocal microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany) connected to a digital camera diagnostic instrument operated by I.A.S. software of Delta Systems Italia (Milan, Italy)

5.5.2.2 Quantitative image analysis

To quantify the immunofluorescence positive area of CD68, PK2, and GFAP in the sciatic nerve and DRG, high-magnification images were captured with a 40x objective at zoom factor 1 using a constant set of acquisition parameters. Six sections were captured from each of five animals per group. The analysis was performed using ImageJ software (version 1.47, http://imagej.nih.gov/ij/index.html, free software) within three boxes of 10^4 μm^2 per section, and a mean value was obtained by combining values from all three boxes. To quantify the immunofluorescence positive area of CD68, PK2, and GFAP in the spinal cord, six L4-

L6 immunofluorescence high-magnification images of the dorsal horns were captured as described above, from each of five animals per group. Quantification was performed within three boxes of 10^4 μm^2 per section that were placed in the lateral, central, and medial areas of the dorsal horns, and a mean value was obtained by combining values from the three boxes.

5.6 *In vitro* Experiments

5.6.1 Primary sensory DRG neurons preparation and culture

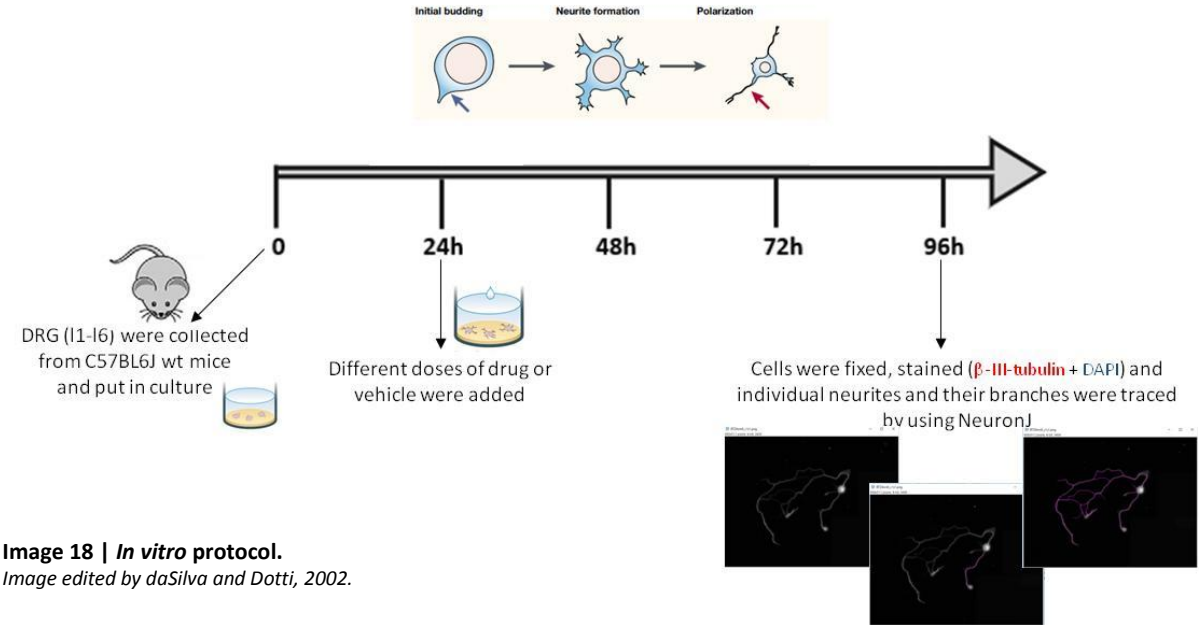


Image 18 | *In vitro* protocol.
Image edited by daSilva and Dotti, 2002.

DRG neurons usually grow *in vitro* as a monolayer and the adhesion to the substrate is absolutely essential for their viability and their ability to extend neurites, therefore we used 12mm glass coverslips (neurite outgrowth) or 24-wells plate (mRNA) incubated at room temperature for 2h with 0.01% poly-L-lysine (Sigma-Aldrich, Italy) and for additional 2h with 1:20 laminin 1mg/ml (Sigma-Aldrich). Lumbar (L1-L6) DRG were dissected from 9 weeks old healthy C57BL/6J male mice as previously described. Ganglia were cleaned from the connective tissue and incubated in Liberase Blendzyme 1 (9 mg/100 ml DMEM, Roche) for 60 min, at 37°C, 5%CO_2. After washing with PBS, Trypsin-EDTA (Invitrogen) was added and samples were incubated at 37°C, 5%CO_2 for 15 min. TNB medium (Biochrom) containing L-glutamin (Invitrogen), penicillin G sodium, streptomycin sulfate (Invitrogen), and Protein-Lipid-Complex (Biochrom) was used for washing. After mechanical dissociation with a fire-polished Pasteur pipette, the resulting cell suspension was centrifuged at 500 rpm through a 3.5% BSA gradient (Sigma-Aldrich) for 10 min. The pellet obtained was washed using TNB medium and centrifuged for 5 min at 760 rpm. At this point cells were resuspended in TNB medium + murine NGF (25ng/ml, Alomone Labs). Neurons were plated on coverslips (for the neurite outgrowth assay) or 24-well plates (mRNA extraction, RT-qPCR), previously coated with poly-L-lysine and laminin as described above. Neurons were cultured in TNB medium with murine NGF (25ng/ml, Alomone Labs) at 37°C in 5% CO_2 for 24h.

After this time span, cells were observed using a light microscope in order to assess cell health and the presence of first neurites; different doses of the chemotherapeutic drugs alone or in association with PC1, PC1 alone and the vehicle were added:

- ***Bortezomib***: 4, 6, 8 and 10nM
- ***Bortezomib 6nM + different doses of PC1***: BTZ +PC1 50nM, BTZ+PC1 100nM, BTZ+PC1 250nM, BTZ+PC1 500nM, BTZ+PC1 1µM
- ***Vincristine***: 0.01, 0.1, 1, 5, 10, 50, 100 nM and 1, 5, 10, 20, 50, 100µM
- ***Vincristine + PC1***: VCR 0.01nM+ PC1 250nM, VCR 0.1nM+ PC1 250nM, VCR 1nM+PC1 250nM and VCR 50nM+PC1 250nM.
- ***PC1***: 50, 100, 250, 500 nM and 1 µM
- ***Vehicle***: Since all the drugs were dissolved in PBS and diluted in TNB medium+ murine NGF (25ng/ml), this solution was used as control (vehicle treated cells).

5.6.2 Immunohistochemical staining and neurite outgrowth assay

After 96 h in culture (72h of drug incubation), DRG neurons were fixed in 4% PFA (10 minutes incubation, room temperature) and subsequently washed with PBS. Cells were permeabilized using 0.1% TX-100 (3min incubation), washed with PBS and then aspecific sites were blocked with Blocking Buffer (1%BSA in PBS) for 30 minutes.

Cells were incubated with a Neuron specific β-III tubulin (TuJ1) monoclonal mouse antibody (1:1000, R&D system) for an hour at room temperature. Neurons were then labeled with chicken anti-mouse IgG secondary antibody, Alexa Fluor™ 594 (30 minutes incubation, 1:1000, ThermoFisher Scientific) to visualize neurites. DAPI (1µg/mL ,Tocris Bioscience) was used to stain cell nuclei. Coverslips were mounted using Mowiol 40-88 (Sigma Aldrich).

For the PK2 localization cells were incubated with the Tuj1 monoclonal mouse antibody and with a rabbit polyclonal to Prokineticin 2 antibody (1:400, Abcam) overnight at 4°C. Neurons were then labelled with a Donkey anti-rabbit IgG secondary antibody, Alexa Fluor™ 488 (30 minutes incubation, 1:400, ThermoFisher Scientific) and with with chicken anti-mouse IgG secondary antibody, Alexa Fluor™ 594 (30 minutes incubation, 1:1000, ThermoFisher Scientific).

Labeling control (sample section incubated in all of the buffers and detergents used in the experiment but no antibodies) were performed before starting the experiments. The secondary antibody control (primary antibody was replaced with the same amount of BSA 1%) was included in each experiment.

Digitalized images of randomly chosen areas of the coverslip were taken using an Axio Imager microscope (Carl Zeiss) with 20X or 63x objectives. Images were recorded with a cooled CCD camera (SPOT; Diagnostic Instruments). At least 80 cells were scored per condition and in triplicates of experiments as minimum requirement. Only neurons that were clearly separated from neighboring cells were included for quantification, neurons with no visible process or with only filopodial formations were counted as negative. Cells showing at least one neurite with total neurite length of at least one cell diameter were counted. Quantification was performed using or ImageJ software and NeuronJ plugin (http://www.imagescience.org/meijering/software/ neuronj/; Meijering et al., 2004).

5.6.3 RNA extraction and RT-qPCR

In order to measure expression levels of the Prokineticin system (PK2, PK-R1, PK-R2), cytokines (IL-1β, TNF-α, IL-6, IL-10), GFAP and ATF3 in the cell cultures, total RNA from cells treated with the following conditions:

- Vehicle
- VCR 1nM
- VCR 1nM+PC1 250nM
- PC1 250nM

was extracted and RT-qPCR for the genes of interest (***Table 2***) was performed as previously mentioned (3.5.1).

5.7 Data analysis

Results are expressed as mean ± SEM.

Data were tested for equal variance before performing the statistical analysis.

Data obtained from pain related behavioural tests were analyzed using Two way-ANOVA, considering the type of treatment and the time as factors. Baseline values (day 0), i.e. responses before neuropathy induction, were not included in the analysis. If an overall test comparing group was significant, Bonferroni's test was used in the post hoc analysis.

Biochemical results were analyzed, at BTZ day 14 and VCR day 7,using unpaired T-test while at the end of the chemotherapeutic schedule (BTZ day 28 and VCR day 14) data were analyzed by means of One way-Anova, followed by Bonferroni's post hoc test for multiple comparison.

Data from fluorescent immunohistochemistry/immunocitochemistry experiments were analyzed and compared by analysis of variance (One way-ANOVA) and by Bonferroni's multiple comparison test.

The overall significance level was 0.05 for each hypothesis. Each group consisted of at least 5 animals. The group size was chosen on the basis of the results obtained in our previous studies. All statistical analysis were performed using GraphPad Prism 6 Software (San Diego, CA, U.S.A).

6. Results

6.1 Bortezomib induced peripheral neuropathy development and effect of PK-Rs antagonist PC1

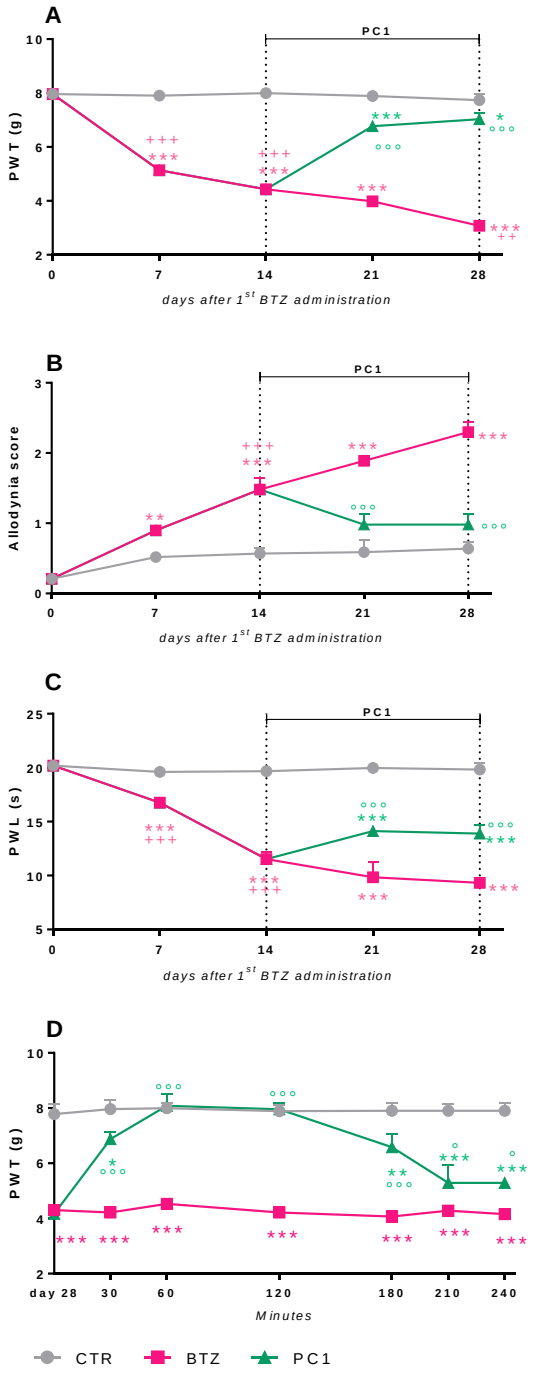


Figure 1 | Pain hypersensitivity due to bortezomib and effect of PK-Rs antagonist PC1

Panels A, B and C show the development of mechanical (panel A) and thermal (panel B) allodynia as well as thermal hyperalgesia (panel C) in mice chronically treated with the chemotherapeutic agent bortezomib (BTZ; 0.4mg/kg, 3 times a week for 4 consecutive weeks) and the effect of chronic PC1 treatment on these painful symptoms. PC1 was administered (s.c. 150µg/kg twice a day) for 14 days starting from BTZ day 14 until the end of the chemotherapeutic schedule, day 28. Panel D shows the effect of a single PC1 injection (s.c. 150 µg/kg) performed at the end of BTZ protocol (day 28). Paw withdrawal thresholds were measured before (day 28) and 30, 60, 120, 180, 210, and 240 minutes after PC1 administration. Data are presented as mean±SEM of 6 mice per group. Statistical analysis was performed by means of Two way-ANOVA analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR; °p<0.05, °°°p<0.001 vs BTZ; $^{++}$p<0.01, $^{+++}$p<0.001 vs the previous BTZ time point.

Before BTZ administration baseline thresholds (day 0) to mechanical and thermal stimuli were similar in all animals. As shown in **Figure 1**, bortezomib administration induced in mice the development of hypersensitivity characterized by the presence of mechanical and thermal allodynia (**panel A** and **B,** respectively) as well as thermal hyperalgesia (**panel C**). A worsening of the pain symptomatology was related to the injected cumulative BTZ dose, in fact 14 days after the first BTZ administration (BTZ cumulative injected dose, 2.4 mg/kg), mechanical and thermal thresholds of BTZ mice were already significantly different from those of BTZ mice at day 7, and a further impairment of mechanical allodynia (**panel A**) was evident at the end of the BTZ protocol, that is day 28 (BTZ cumulative injected dose 4.8 mg/kg).

At day 14, in presence of a well-established allodynic and hyperalgesic state, a group of BTZ-mice started the chronic treatment with the PK-Rs antagonist PC1, that was s.c. injected at the dose of 150µg/kg two times/day until day 28, simultaneously to BTZ schedule. As controls, a group of BTZ-mice was treated with the vehicle.

Already after seven days (BTZ day 21), chronic treatment with the PK-Rs antagonist ameliorated both mechanical (**panel A**) and thermal (**panel B**) allodynia as well as thermal hyperalgesia (**panel C**), and its positive effect was present until the end of the BTZ protocol.

The acute effect of a single PC1 injection on mechanical allodynia was tested at the end of the BTZ protocol (**panel D**). PC1 was able to rapidly counteract mechanical allodynia, and its effect was maximal between 60 and 120 min and then progressively decreased, although a significant difference from BTZ mice was still present 240 min after PC1 administration.

Since we demonstrated in our previous study (Castelli et al., 2016) that thermal and mechanical sensitivity of healthy (control) mice treated with PC1 were not altered and considering the recent European guidelines regulating animal research, which suggest to minimize the number of animals, this experimental group was not included in the present study.

Given the fact that PC1 chronic treatment was effective in alleviating painful symptoms, the activation of PK system and the presence of neuroinflammation were evaluated in

the main stations of pain transmission (sciatic nerve, DRG and spinal cord) before starting PC1 chronic treatment, that is 14 days after the first BTZ injection (BTZ day 14, corresponding to BTZ cumulative injected dose of 2.4 mg/kg) and the end of BTZ/PC1 protocol (BTZ day 28, corresponding to BTZ cumulative injected dose of 4.8 mg/kg).

6.1.1 Prokineticin system activation in sciatic nerve

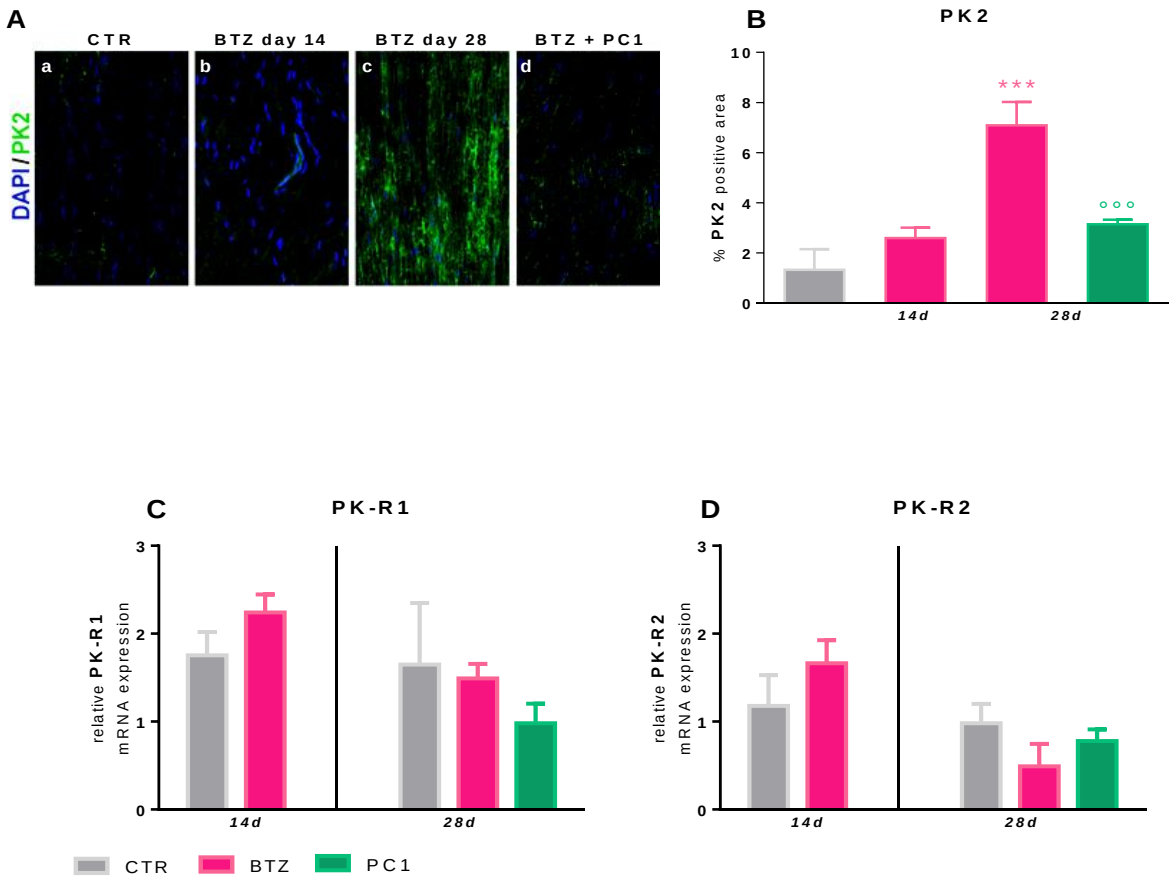


Figure 2 | Prokineticin system activation in the sciatic nerve.
Panel A shows representative images (a-d) of PK2 immunofluorescence signal in the sciatic nerve sections of CTR (picture a), BTZ day 14 (picture b), BTZ day 28 (picture c), and BTZ + PC1 day 28 mice (picture d). Cell nuclei were counterstained with DAPI (blue fluorescence). Quantitative analysis of PK2 signal (panel B) was computed as integrated optical density for arbitrary areas (6 sections per animal, 5 animals per group). Scale bar =20µM One-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. ***p < 0.001 vs CTR; °°°p < 0.001 vs BTZ day 28.
Panels C and D show mRNA levels of PK-R1 and PK-R2 respectively, measured by Real Time-qPCR, 14 days after the first BTZ administration and at the end of BTZ protocol (day 28). Data represent mean±SEM of 6 mice/group. At day 14 statistical analysis was performed by means of unpaired T- test while at day 28 by means of One way-Anova analysis of variance followed by Bonferroni's post test.

PK2 levels in the sciatic nerve were assessed by immunofluorescence analysis. As shown in **Figure 2**, 14 days of BTZ treatment did not induce any change in PK2 protein levels (**panel A, picture b**), as demonstrated by the quantification of PK2 positive area percentage (**panel B**). However, at the end of the chemotherapeutic schedule (BTZ day 28), PK2 levels were increased, as shown by the representative immunofluorescence images (**panel A, picture c**) and by the quantification of PK2 positive area (**panel B**). PC1 chronic treatment significantly reduced PK2 signal (**panel A, picture d** and **panel B**). At the same experimental time points (BTZ day 14 and 28), PK-R1 (**panel C**) and PK-R2 (**panel D**) expression levels were not affected by BTZ nor by BTZ+PC1.

6.1.2 Effect of PK-Rs antagonism on sciatic nerve neuroinflammation

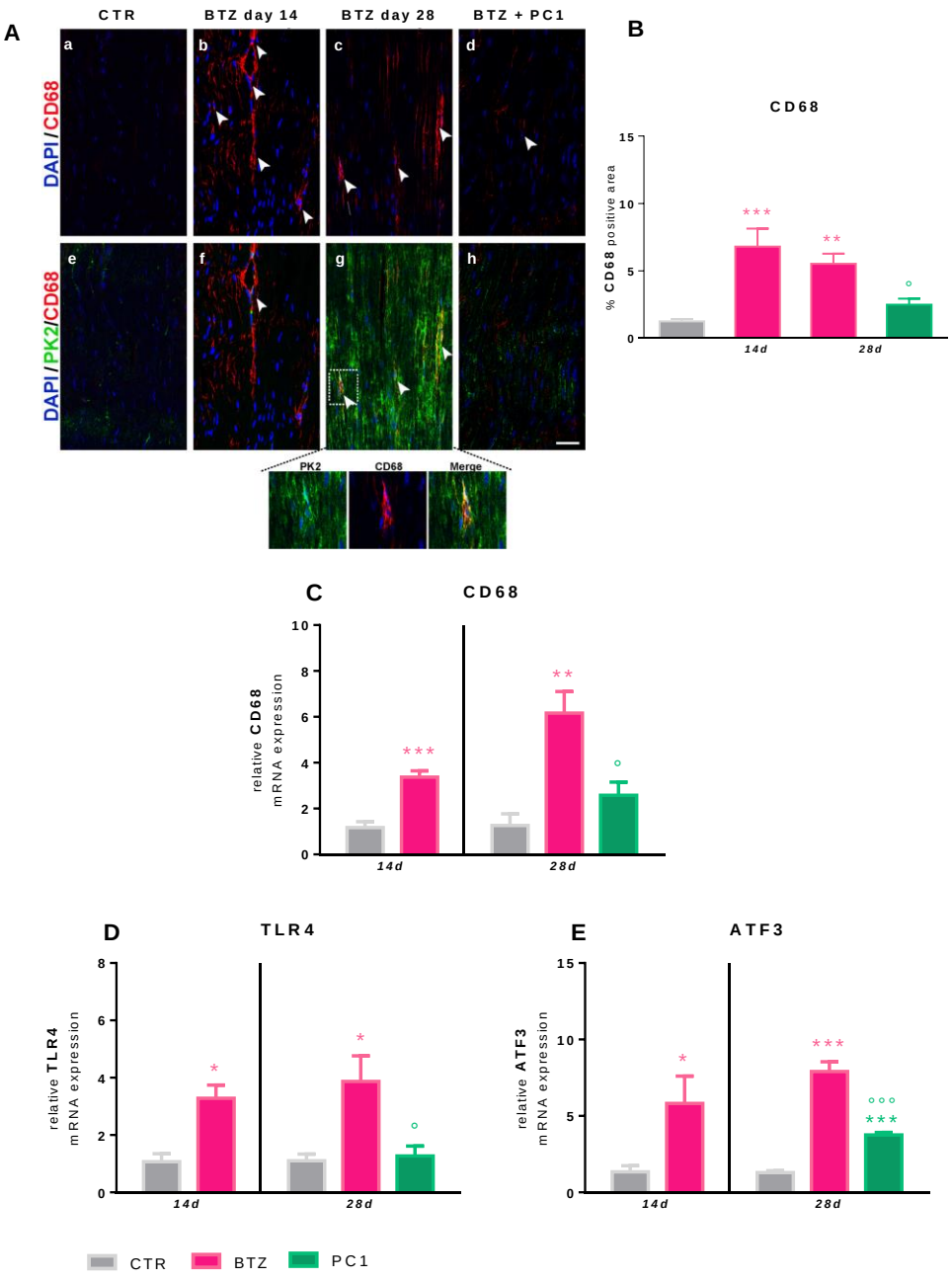


Figure 3 | Neuroimmune activation in the sciatic nerve.
Panel A shows representative images of CD68 immunofluorescence (fig a –d, white arrowhead) and the colocalization (pictures e-h; yellow, white arrowhead) of PK2 (green) with CD68 (activated macrophages, red) in sciatic nerve sections of CTR (pictures a and e), BTZ day 14 (pictures b and f), BTZ day 28 (pictures c and g), and BTZ + PC1 (pictures d and h) mice. Panel B shows the quantification of CD68 as positive area percentage. Quantitative analysis of CD68 signal was computed as integrated optical density for arbitrary areas (6 sections per animal, 5 animals per group). Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar =30µM One-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. **p < 0.01, ***p < 0.001 vs CTR; °p < 0.05 vs BTZ day 28.
Expression levels of the neuroimmune markers CD68 (panel C), TLR4 (panel D) and of the markers of tissue damage/cell stress, ATF3 (panel E) determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at day 14 and day 28 after the 1st BTZ injection). Data are presented as mean±SEM of 6 mice/group. At day 14, statistical analysis was performed by means of T-test, while at day 28 by means of One way-ANOVA followed by Bonferroni's post-test. *p<0.05,**p<0.01, ***p<0.001 vs CTR; °p<0.05, °°°p<0.001 vs BTZ day 28.

In order to better investigate the immune/inflammatory components, we assessed the macrophage/Toll Like Receptor 4 (TLR4) expression. It is known the recruitment and infiltration of activated macrophages in the peripheral nerves and DRG has a key role in the development of CIPN and that PK2 is able to induce the macrophage to migrate and to acquire a pro-inflammatory phenotype, so we decided to evaluate both the distribution and the expression levels of CD68 (Cluster of Differentiation 68) which is routinely used as a marker of macrophages and of TLR4, which activation is linked to pro-inflammatory cytokines modulation.

As illustrated in **figure 3**, at day 14 (14d), that is before starting PC1 chronic treatment, an increase of the marker of activated macrophages, CD68 was present as shown in the immunofluorescence images of **panel A** (**picture b**) and its quantification (**panel B**). Also CD68 expression resulted upregulated at the same time

point (**panel C**). Simultaneously (day 14), we also observed increased mRNA levels of TLR4 (**panel D**) and of the marker of cell stress/injury ATF3 (**panel E**).
At the end of the BTZ schedule (day 28), mice treated with the chemotherapeutic drug still show an upregulation of CD68 (**panel A, picture c** and **panel B** and **C**). Interestingly, immunofluorescence double-staining images (**panel A, picture g**) illustrate that PK2 immunoreactivity partially colocalizes with the CD68 signal (**picture g enlargement**).
After 14 days of chronic treatment (BTZ day 28), PC1 was able to contrast macrophage recruitment and activation, as demonstrated by the reduction of CD68 (**panel A, picture d; panels B** and **C**) and TLR4 levels (**panel D**). Also cell stress/ injury was positively modulated by PC1 administration, as shown by the reduced ATF3 expression levels (**panel E**).

In the same nervous tissue, at day 14, we detected in BTZ-mice a pro-inflammatory profile, **figure 4**, characterized by an increase in the expression levels of IL-1β (**panel A**) and IL-6 (**panel C**) while no changes were detected in TNF-α (**panel B**) and IL-10 (**panel D**) mRNA. By increasing the BTZ injected cumulative dose, at day 28 (BTZ cumulative injected dose 4.8mg/kg), a more pronounced pro-inflammatory profile was evident. In fact, at the end of the chemotherapeutic schedule (BTZ day 28) all the analyzed pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) resulted upregulated (**panels A, B** and **C**) and a significant decrease in the expression levels of the anti-inflammatory cytokine IL-10 was also present (**panel D**). PC1 chronic treatment was able not only to reduce IL-1β (**panel A**) and IL-6 (**panel C**) but also to prevent TNF-α (**panel B**) mRNA increase. Moreover, PK-Rs antagonist partially prevented decrease of IL-10 expression (**panel D**).

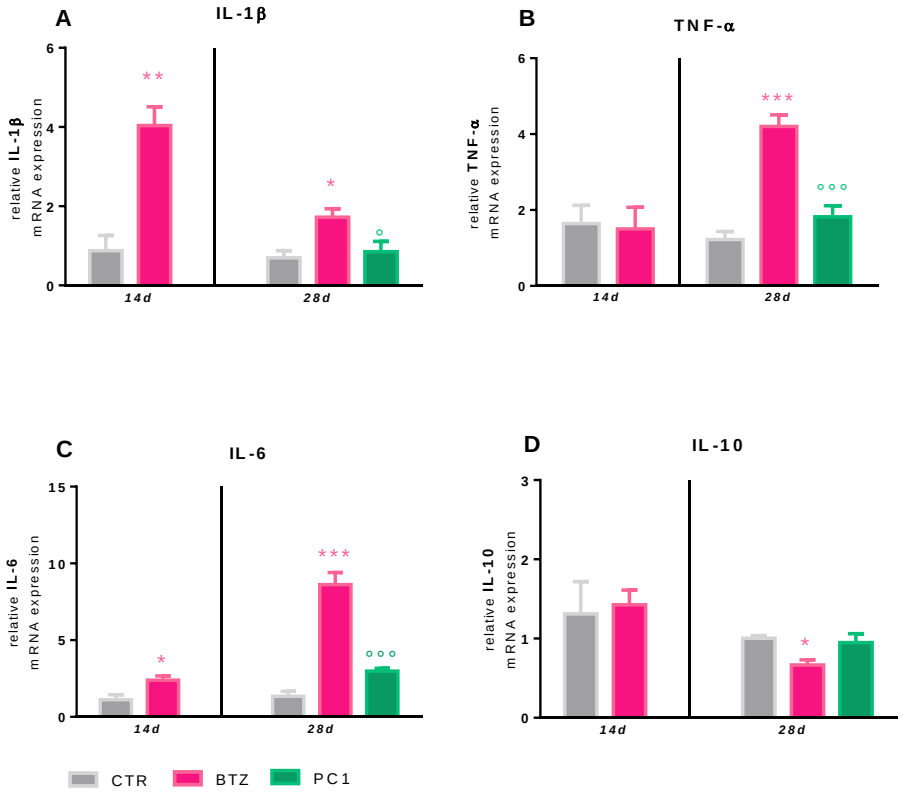


Figure 4 | Cytokine expression levels in the sciatic nerve.
Panels A, B and C represent respectively the mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6; panel D reports those of the anti-inflammatory cytokine IL-10. Analysis were performed 14 days after the first BTZ administration, before starting PC1 treatment (CTR and BTZ groups), and at the end BTZ/ BTZ+ PC1 protocol (CTR, BTZ, BTZ+ PC1 groups). mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals at the same time point. Data are presented as mean±SEM of 6 mice/group. At day 14 statistical analysis was performed by means of unpaired T- test while at day 28 by means of One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR (at the same time point); °p<0.05, °°°p<0.001 vs BTZ day 28.

6.1.3 Prokineticin system activation in dorsal root ganglia

PK system activation in DRG is shown in **figure 5**. At BTZ day 14 no changes in PK2 immunoreactivity (**panel A, picture b** and **panel B**) or in its expression (**panel C**) were observed, and also PK-Rs (**panels D** and **E**) were not altered.

However, at the end of BTZ schedule (day 28), a consistent increase of PK2 signal as shown by immunofluorescence representative images (**panel A, picture c**) and its quantification (**panel B**) as well as an upregulation of PK2 (**panel C**) and its receptors (**panel D** and **panel E**) expression was present in the DRG of BTZ-mice. PC1 was able to prevent the increase of both PK2 (**panel A, picture d; panels B** and **C**) and of PK-R1 (**panel D**). No effect was detected on PK-R2 mRNA levels (**panel E**).

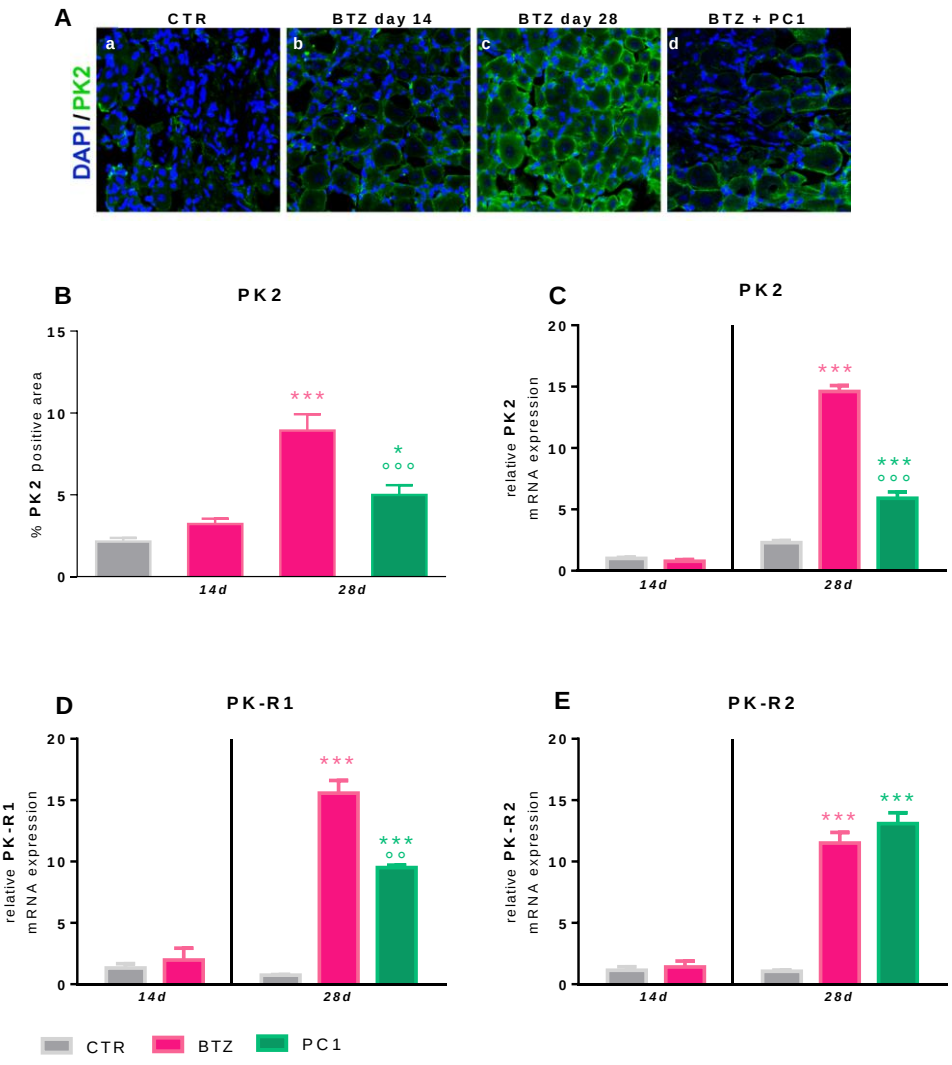


Figure 5 | Prokineticin system activation in dorsal root ganglia. Representative images of PK2 immunofluorescence signal in DRG sections of CTR (picture a), BTZ day 14 (picture b), BTZ day 28 (picture c), and BTZ+PC1 (picture d) mice. Quantitative analysis of PK2 signal (panel B) was computed as integrated optical density for arbitrary areas (6 sections per animal, 5 animals per group). Scale Bar=30μM One-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. *p < 0.05, ***p < 0.001 vs CTR; °°°p < 0.001 vs BTZ day 28.

Panels C, D and E illustrate mRNA levels of PK2, PK-R1 and PK-R2 respectively, measured by Real Time-qPCR, 14 days after the first BTZ administration in CTR and BTZ mice and at the end of the chemotherapeutic schedule (day 28) in CTR, BTZ and BTZ + PC1. mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-ncreases over the levels of CTR animals (at the same time point).

Data are presented as mean±SEM of 6 mice/group. At day 14, statistical analysis was performed by means of unpaired T-test while at day 28 by One way-Anova analysis of variance followed by Bonferroni's post test. ***p<0.001 vs vehicle/CTR (at the same time point); °°p<0.01, °°°p<0.001 vs BTZ day 28.

6.1.4 Effect of PK-Rs antagonism on dorsal root ganglia neuroinflammation

Also in DRG, BTZ induced a rapid macrophage recruitment and activation that was already evident at day 14. At this time point, as shown in **figure 6**, we observed in DRG sections of BTZ-mice an increase of CD68 protein levels as reported by immunofluorescence pictures (**panel A, picture b**) and the quantification of CD68 positive area (**panel B**). Also CD68 (**panel C**) and TLR4 (**panel D**) expression resulted upregulated at this time while no alteration in ATF3 mRNA was detected (**panel E**). At longer time point (BTZ day 28)

immunofluorescence double-staining images show, in BTZ-mice, the partial association between CD68 and PK2 signal (**panel A, picture g enlargement**).

PK2 activation was reduced in PC1-treated mice and its association with CD68 was not detectable anymore (**panel A, picture h**). PC1 treatment was also able to normalize protein and mRNA levels of CD68 (**panel A, picture d**; **panels B** and **C**) and TLR4 (**panel D**) but it had no effect on the increased ATF3 expression (**panel E**).

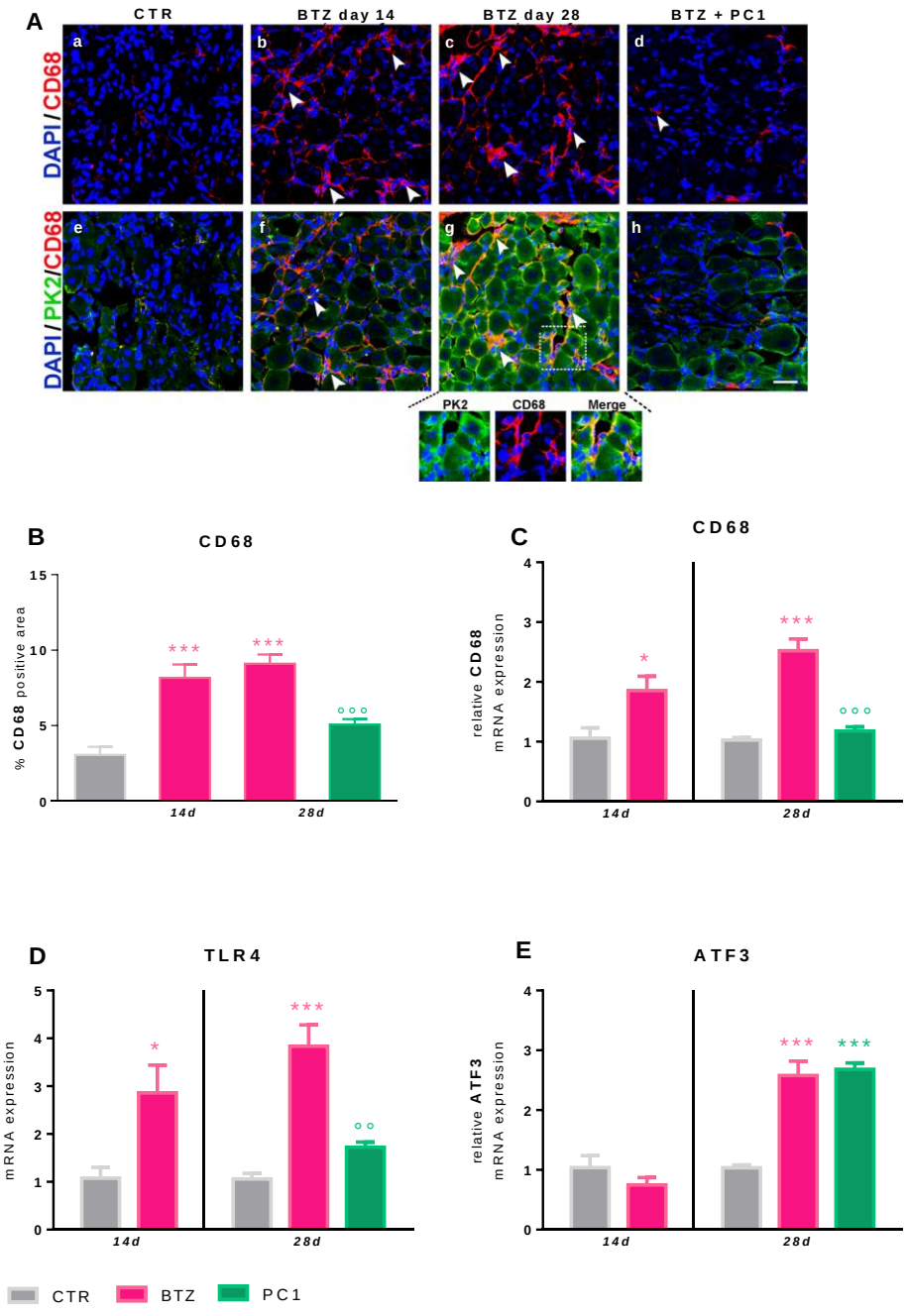


Figure 6 | Neuroimmune activation in dorsal root ganglia.
Fourteen days after the first BTZ injection and at the end of BTZ/BTZ+PC1 protocol (BTZ day 28), the effect of PC1 treatment on macrophage activation in the DRG was assessed. Panel A shows representative images of CD68 immunofluorescence (pictures a –d, white arrowheads) and the colocalization (pictures e-h; yellow, arrowhead) of PK2 (green) with CD68 (activated macrophages, red) in DRG sections of CTR (pictures a and e), BTZ day 14 (pictures b and f), BTZ day 28 (pictures c and g), and BTZ + PC1 (pictures d and h) mice. Panel B shows the percentage of CD68 positive area in the DRG of CTR, BTZ day 14, BTZ day 28 and BTZ + PC1 day 28 mice. Scale bar=30μM One-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. ***p < 0.001 vs CTR; °°°p < 0.001 vs BTZ day 28. Expression levels of CD68 (panel C), TLR4 (panel D) and of the marker of tissue damage/cell stress, ATF3 (panel E) determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 6 mice/group. At day 14 statistical analysis was performed by means of unpaired T- test while, at day 28, One Way-ANOVA followed by Bonferroni's test for multiple comparisons were used. *p<0.05, ***p<0.001 vs CTR; °°p<0.01, °°°p<0.001 vs BTZ day 28.

In the same nervous tissue, at day 14 (14d) BTZ-mice did not show changes in IL-1β mRNA, however an alteration of different cytokines was already evident. In fact, as shown in **figure 7**, increased expression of TNF-α (**panel B**) and IL-6 (**panel C**) and a significant decrease of IL-10 (**panel D**) were observed. At day 28 (28d), a more pronounced pro-inflammatory profile characterized by an upregulation of all the analyzed

pro-inflammatory cytokines (IL-1β, TNF-α and IL-6, **panels A,B** and **C** respectively) was present in DRG of BTZ-mice. PC1 chronic treatment, probably by preventing macrophage activation, was able to decrease neuroinflammation by avoiding the upregulation of IL-1β (**panel A**), and reducing the expression of TNF-α (**panel B**) and of IL-6 (**panel C**). A slight trend toward increased IL-10 mRNA (**panel D**), probably due to a physiological response to neuroinflammation, was present in DRG of BTZ-mice; PC1 chronic treatment was able to mitigate this trend.

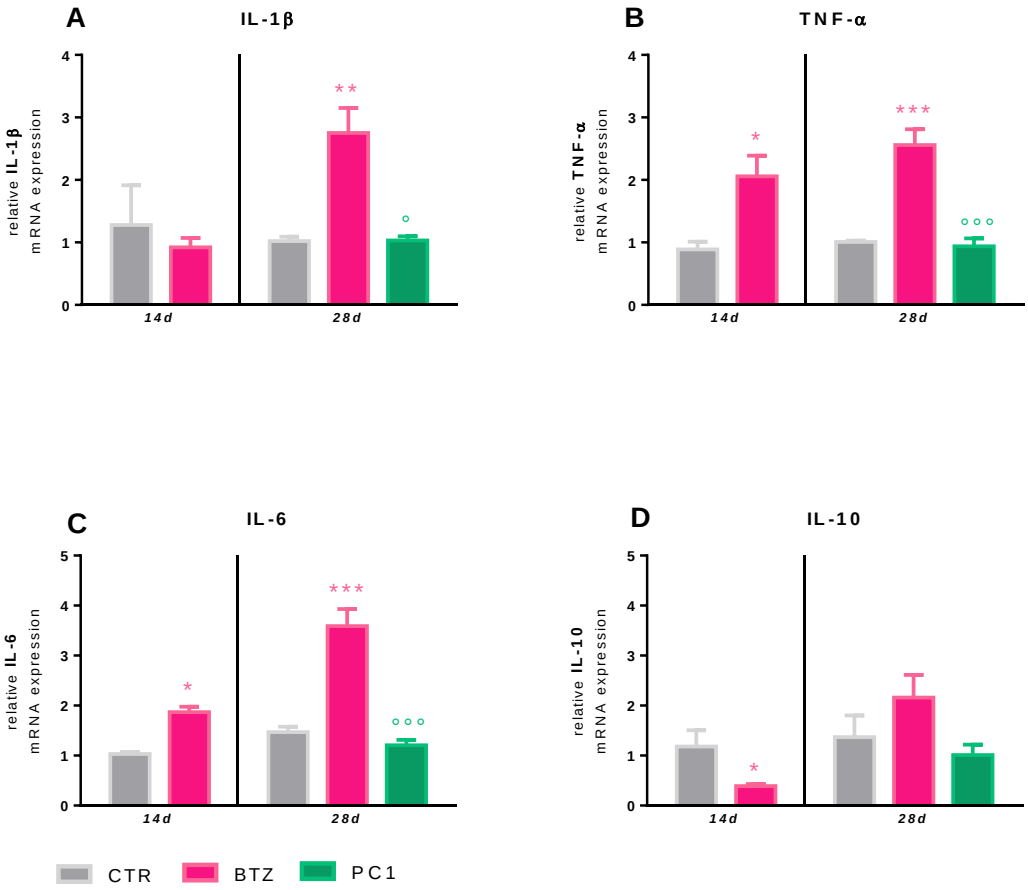


Figure 7 | Cytokine expression levels in dorsal root ganglia.
Panels A, B and C represent respectively the mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 ; panel D reports that of the anti-inflammatory cytokine IL-10. Analysis were performed 14 days after the first BTZ administration, before starting PC1 treatment (CTR and BTZ groups), and at the end BTZ/ BTZ+ PC1 protocol (CTR, BTZ, BTZ+ PC1 groups). mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 6 mice/group. At day 14 statistical analysis was performed by means of unpaired T- test while at day 28 by means of One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR (at the same time point); °p<0.05, °°°p<0.001 vs BTZ day 28.

6.1.5 Prokineticin system activation in the spinal cord

As shown in **figure 8**, 14 days (14d) after the first BTZ administration, no alteration in the PK system was detected in the spinal cord. In fact, at this time point PK2 immunofluorescence (**panel A, picture b**; **panel B**) and PK receptors (**panel C** and **D**) mRNA were not altered in BTZ-mice and appeared comparable to those of control mice. However, at the end of the chemotherapeutic schedule (28d), BTZ induced an increase in PK2 signal in the dorsal horns of the spinal cord, as shown in the immunofluorescence picture (**panel A, picture c**) and its quantification (**panel B**). An increase of PK-R1 (**panel C**) and PK-R2 (**panel D**) expression was also present at this time point. Chronic therapeutic treatment with PC1 was able to prevent PK system (PK2 and PK-Rs) upregulation in the spinal cord (**panel A, picture d**; **panels B**, **C** and **D**).

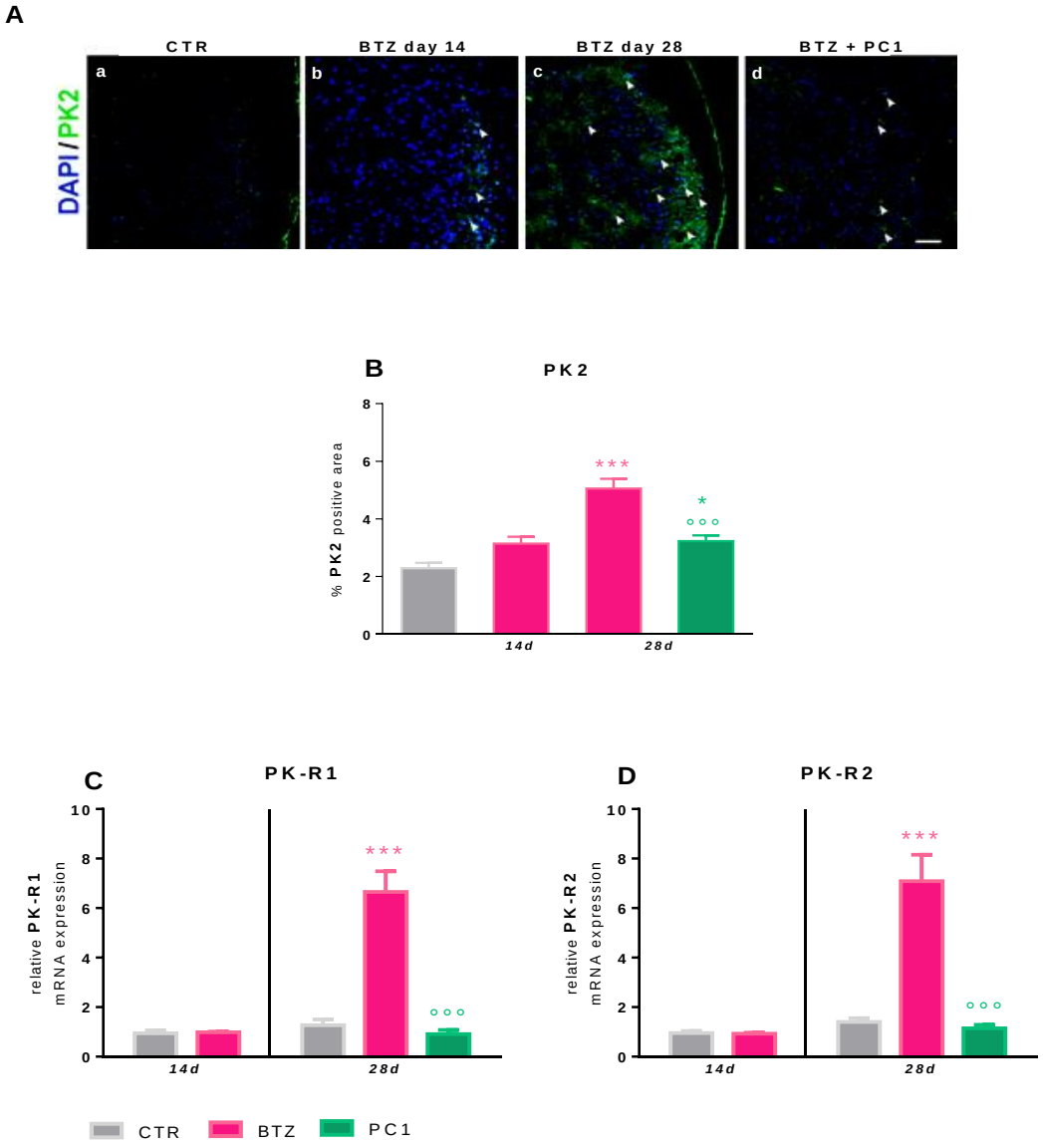


Figure 8 | Prokineticin system activation in spinal cord.
Panel A (pictures a - d) shows representative images of PK2 immunofluorescence signal (white arrowhead) in spinal cord sections of CTR (picture a), BTZ day 14 (picture b), BTZ day 28 (picture c) and BTZ + PC1 day 28 (picture d) mice. Quantitative analysis of PK2 signal (panel B) was computed as integrated optical density for arbitrary areas (6 sections per animal, 5 animals per group), scale bar=50µM. One-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. *p < 0.05, ***p < 0.001 vs CTR; °°°p < 0.001 BTZ day 28.
mRNA levels of PK-R1 and PK-R2 are shown in panels C and D respectively, measured by Real Time-qPCR, 14 days after the first BTZ administration (CTR and BTZ mice) and at the end of the BTZ protocol (day 28) in all experimental groups. mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold increases over the levels of CTR animals (at the same time point). Data represent mean ± SEM of 6 mice/group. At day 14, statistical analysis was performed by means of T- test while at day 28 by means of One way-ANOVA followed by Bonferroni's post-test. ***p<0.001 vs CTR; °°°p<0.001 vs BTZ day 28.

6.1.6 Effect of PK-Rs antagonism on spinal cord neuroinflammation

As shown in **figure 9**, at the beginning (day 14) the spinal cord was marginally affected by BTZ. In fact, at this time, the only alteration observed was an increase of the astrocytes marker GFAP immunofluorescence signal (**panel A picture f**) and its quantification (**panel B**), while the microglia marker CD68 (**panel A picture b, panel C**), TLR4 (**panel D**) and ATF3 (**panel E**) were not modified by BTZ treatment.

However, at the end of the chemotherapeutic schedule (BTZ 28d), also the spinal cord underwent to some changes. In fact, at this time point, also CD68 (**panel C**), TLR4 (**panel D**) and ATF3 (**panel E**) mRNA was upregulated. However, as shown in **panel A, picture g**, immunofluorescence double-staining images illustrate how, in the spinal cord of BTZ-mice at day 28, PK2 signal was associated with GFAP but not to CD68 (**panel A**, **picture c**).

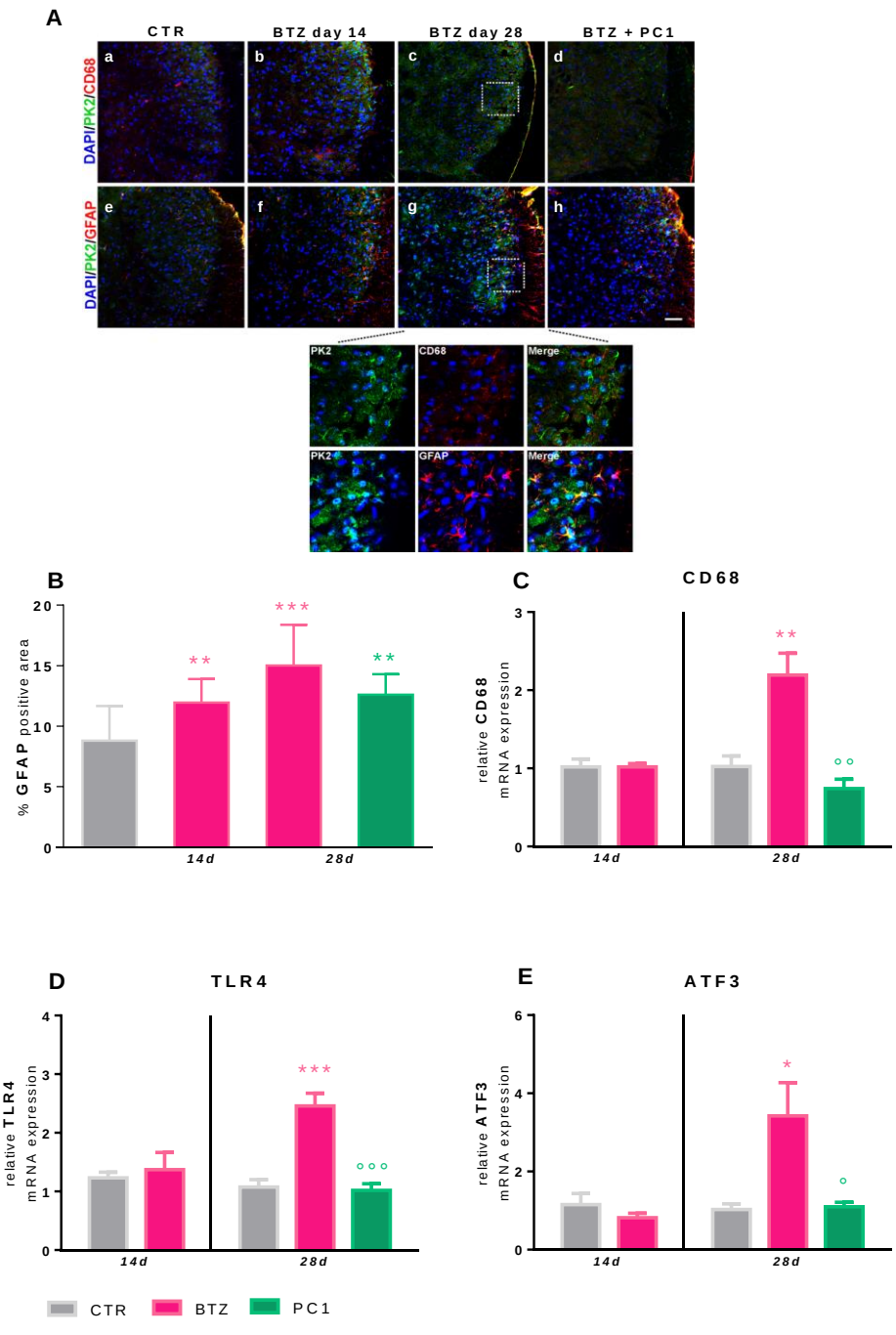


Figure 9 | Neuroimmune activation in spinal cord. Panel A (pictures a-d) shows the immunofluorescence double staning of PK2 (green) and CD68 (microglia, red). In the same panel (pictures e-h) there are representative images of the colocalization (yellow) of PK2 (green) with GFAP (astrocytes, red) in the spinal cord of CTR (picture a and e), BTZ day 14 (picture b and f), BTZ day 28 (picture c and g), and BTZ + PC1 day 28 (picture d and h) mice. Quantitative analysis of GFAP positive signal (panel B) was computed as integrated optical density for arbitrary areas (6 sections per animal, 5 animals), scale bar=50µM . One way-ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. **p < 0.01, ***p < 0.001 vs CTR.
mRNA levels of CD68, TLR4 and ATF3 are shown in panel C, D and E respectively. Expression levels were measured by Real Time-qPCR and they were expressed in relation to GAPDH and presented as fold increases over the levels of CTR animals (at the same time point). Data represent mean±SEM of 6 mice/group. At day 14, statistical analysis was performed by means of T-test, while at day 28 by means of One way-ANOVA followed by Bonferroni's post-test.
*p<0.05,**p<0.01, ***p<0.001 vs CTR; °p<0.05, °°p<0.01, °°°p<0.001 vs BTZ day 28.

In accordance with the decrease of PK2 signal after PC1 treatment (**figure 8 panels A** and **B**), also PK2/GFAP colocalization disappeared in PC1-treated animals (**panel A picture h**). PC1 was unable to prevent GFAP upregulation (**panel B**) however, it prevented the immune/glial activation and cell stress/damage in the lumbar spinal cord by maintaining low levels of CD68 (**panel C**), TLR4 (**panel D**) and ATF3 (**panel E**).

In the central nervous system, as shown in **figure 10**, at day 14 no alterations in the expression of the analyzed cytokines (**panels A, B, C** and **D**) were detected. However, at the end of BTZ schedule, we observed an increase of the pro-inflammatory cytokine IL-1β (**panel A**) and a decrease in the levels of the anti-inflammatory cytokine IL-10 (**panel D**). This cytokine production was concurrent with the alterations shown in **figure 9**. No changes in mRNA of TNF-α (**panel B**) and IL-6 (**panel C**) were present also at this time. PC1 chronic administration was able to preserve a correct pro-/anti-inflammatory (**panels A** and **D**) cytokine balance in the spinal cord.

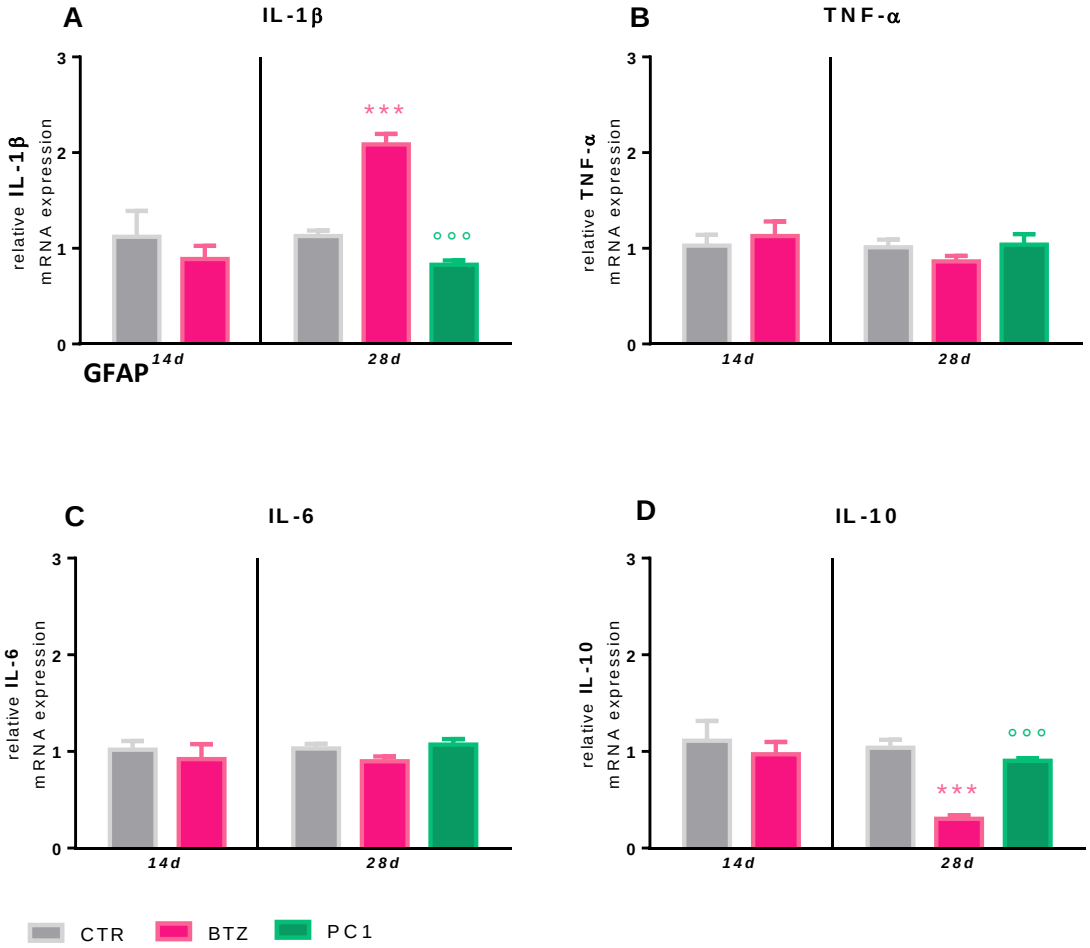


Figure 10 | Cytokine expression levels in spinal cord.
Panels A, B and C represent respectively the mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6; panel D reports that of the anti-inflammatory cytokine IL-10. Analysis were performed 14 days after the first BTZ administration, before starting PC1 treatment (CTR and BTZ groups), and at the end BTZ/ BTZ+ PC1 protocol (day 28; CTR, BTZ, BTZ+ PC1 groups). mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 6 mice/group. At day 14 statistical analysis was performed by means of unpaired T- test while at day 28 by means of One way-Anova analysis of variance followed by Bonferroni's post test. ***p<0.001 vs vehicle/CTR (at the same time point); °°°p<0.001 vs BTZ day 28.

6.1.7 Effect of PK-Rs antagonism on multiple bortezomib cycles

After 28 days of BTZ and 14 days of PC1 administration, both treatments were interrupted. As shown in **figure 11**, PK-Rs antagonist continued to exert its positive effect and PC1-mice seemed to show a faster recovery (day 49) than BTZ- mice (day 63).

In order to more closely resemble the clinical situation, where patients are often subjected to several cycle of chemotherapy, at day 84, when BTZ-mice completely recovered and their mechanical thresholds were similar to those of controls, we started to administer to animals previously treated with the chemotherapeutic agent a new BTZ schedule using a protocol identical to the one used for the first BTZ cycle (0.4mg/kg, 3 times a week/4 consecutive weeks).

The second BTZ cycle induced an allodynic effect similar to that observed during the first BTZ cycle, but interestingly, at day 98 (that is 14 days after the beginning of the second BTZ schedule), in mice that had been previously treated with PC1, the allodynic effect induced by BTZ was less intense in comparison with that observed in BTZ-only re-treated mice.

Furthermore, a second PC1 treatment (using the same schedule: 150µg/kg twice a day/14 consecutive days) started at day 98 was able to completely reverse mechanical allodynia as demonstrated by the PWT of PC1-treated mice that were equivalent to those of controls animals at the end of the BTZ/PC1 schedule (day 112).

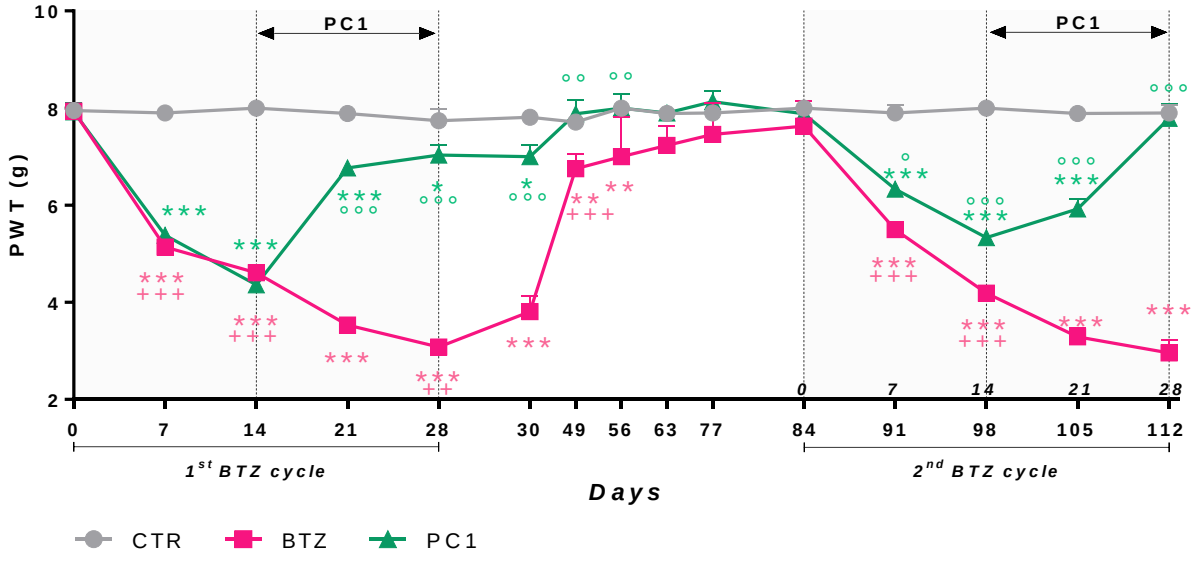
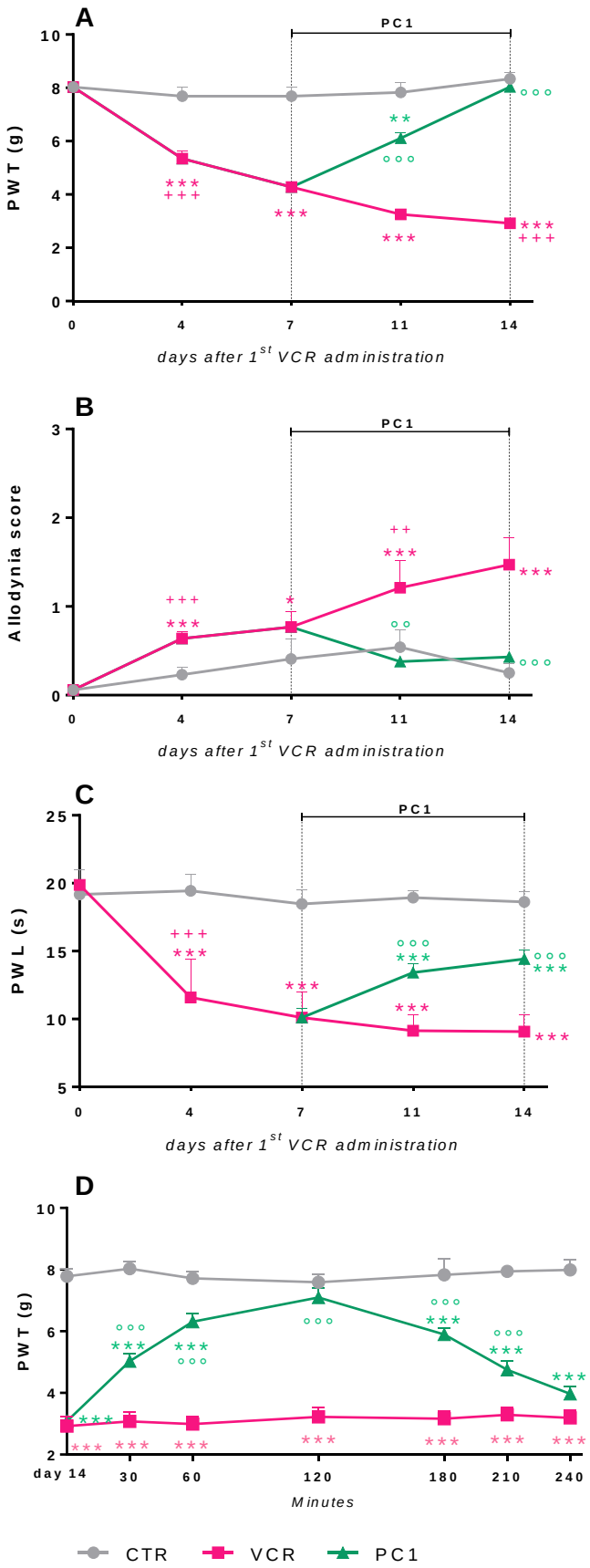


Figure 11 | Mechanical allodynia development during a second BTZ cycle.
Following the interruption of a classical BTZ protocol of 28 days (BTZ 0.4 mg/kg, 3 times week/4 weeks, i.p.) and PC1 (s.c. 150 µg/kg twice day) chronic treatment (from BTZ day 14 to 28), mice progressively recovered from mechanical hypersensitivity. At day 84, in the presence of basal mechanical thresholds, mice that have been previously treated with the chemotherapeutic drug (first BTZ cycle) underwent a second identical treatment with BTZ (BTZ 0.4 mg/kg, 3 times week/4 weeks, i.p.). At day 98, mice previously treated with PC1, started a new chronic treatment with the PK-Rs antagonist (150µg/kg, twice a day for 14 consecutive days, s.c.). All animals were monitored until the end of the second BTZ and PC1 treatment (28 days since the beginning of the second cycle corresponding to 112 days after the first BTZ injection). Data represent mean±SEM of 6 mice/group. Statistical analysis was performed by means of Two way-ANOVA followed by Bonferroni's post-test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR; °p<0.05, °°p<0.01, °°°p<0.001 vs BTZ; $^{++}$p<0.01, $^{+++}$p<0.001 vs BTZ at the previous time point.

6.2 Vincristine induced peripheral neuropathy development and effect of PK-Rs antagonist PC1



As shown in **figure 12**, before VCR administration baseline thresholds (day 0) to mechanical and thermal stimuli were similar in all animals. Vincristine administration induced in mice the development of hypersensitivity characterized by the presence of mechanical and thermal allodynia (**panel A** and **B,** respectively) as well as thermal hyperalgesia (**panel C**).

A worsening of the pain symptomatology was related to the cumulative injected VCR dose, in fact 14 days after the first VCR administration (VCR cumulative injected dose, 1.4 mg/kg), mechanical thresholds of VCR mice were lowered if compared to those of VCR mice at day 11. At day 7, in presence of a well-established allodynic and hyperalgesic state, a group of VCR-mice started the chronic treatment with the PK-Rs antagonist PC1, that was subcutaneously injected at the dose of 150µg/kg two times/day, simultaneously to VCR until day 14. As control, VCR-mice were treated with the vehicle. Already after 4 days of chronic treatment with the PK-Rs antagonist (i.e. VCR day 11), a significant improvement in the responses of PC1-treated mice was evident in comparison to VCR-only animals (**panels A, B** and **C**). Seven days of chronic PC1 treatment (VCR day 14) completely abolished mechanical (**panel A**) and thermal (**panel B**) allodynia. The

Figure 12 | Pain hypersensitivity due to vincristine and effect of PK-Rs antagonist.
Panels A, B and C show the effect of chronic PC1 treatment on mechanical (panel A) and thermal (panel B) allodynia as well as on thermal hyperalgesia (panel C) induced by vincristine (VCR) chronic administration (i.p., 0.1mg/kg, once a day for 14 consecutive days). PC1 was administered (s.c., 150µg/kg twice a day) for 7 days starting from day 7 until the end of VCR schedule, day 14. Panel D shows the effect of a single PC1 injection (s.c. 150 µg/kg) performed at the end of VCR protocol (day 14). Paw withdrawal thresholds were measured before (day 14) and 30, 60, 120, 180, 210 and 240 minutes after PC1 administration.
Data are presented as mean±SEM of 8 mice/group. Statistical analysis was performed by mean of Two way-ANOVA analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR; °°p<0.01, °°°p<0.001 vs VCR mice at day 14; +p<0.05, ++p<0.01, +++p<0.001 vs VCR at the previous time point.

PK-Rs antagonist was also able to significantly counteract thermal hyperalgesia even if the paw withdrawal latencies (PWL) of PC1 treated mice were still different from those of CTR mice (**panel C**).

Also in this case, as for BTZ experiments, we tested the acute effect of a single PC1 injection on mechanical allodynia at the end of the VCR protocol (VCR day 14, **panel D**). PC1 was able to rapidly counteract mechanical allodynia, and its effect started to be detectable already after 30 minutes, even if it was maximal at around 120 min. After this time point the antagonist effect started to decrease and, 240 min after the injection, no significant difference was present anymore between VCR and PC1 treated mice.

6.2.1 Prokineticin system activation in dorsal root ganglia

PK system activation in DRG is shown in **figure 13**. After seven days of VCR chronic administration (i.e. before starting PC1 schedule), PK2 expression (**panel A**) did not show any change in the DRG of mice treated with the chemotherapeutic agent. On the other hand, at the same time point, both PK-Rs resulted upregulated (panels **B** and **C**) in the same tissue. At the end of the chemotherapeutic schedule (VCR day 14) besides altered PK-Rs expression levels, an increase in the mRNA of PK2 in VCR- treated mice was detected. PC1 reduced PK-Rs upregulation (**panels B** and **C**) and to prevent PK2 increase (**panel A**).

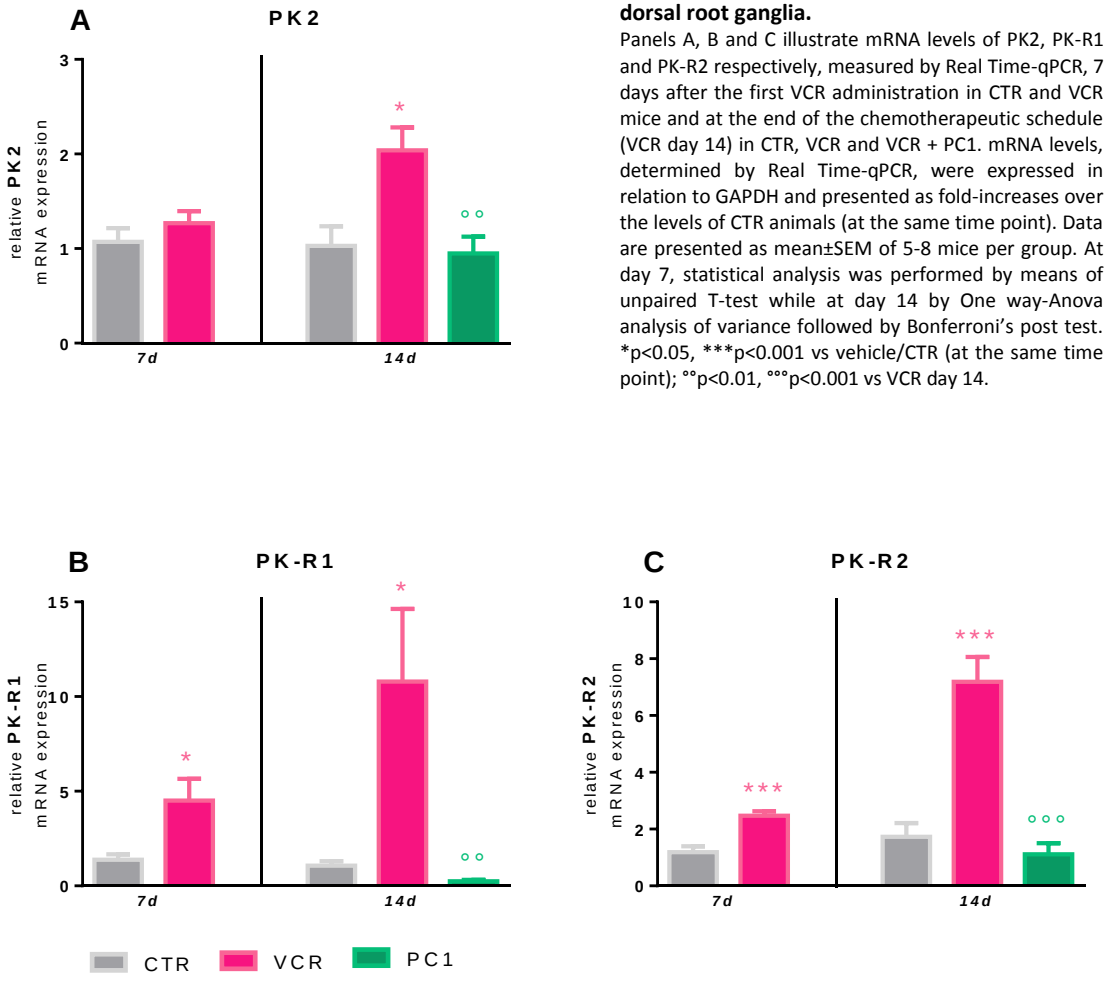


Figure 13 | Prokineticin system activation in dorsal root ganglia.
Panels A, B and C illustrate mRNA levels of PK2, PK-R1 and PK-R2 respectively, measured by Real Time-qPCR, 7 days after the first VCR administration in CTR and VCR mice and at the end of the chemotherapeutic schedule (VCR day 14) in CTR, VCR and VCR + PC1. mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 5-8 mice per group. At day 7, statistical analysis was performed by means of unpaired T-test while at day 14 by One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05, ***p<0.001 vs vehicle/CTR (at the same time point); °°p<0.01, °°°p<0.001 vs VCR day 14.

6.2.2 Effect of PK-Rs antagonism on dorsal root ganglia neuroinflammation

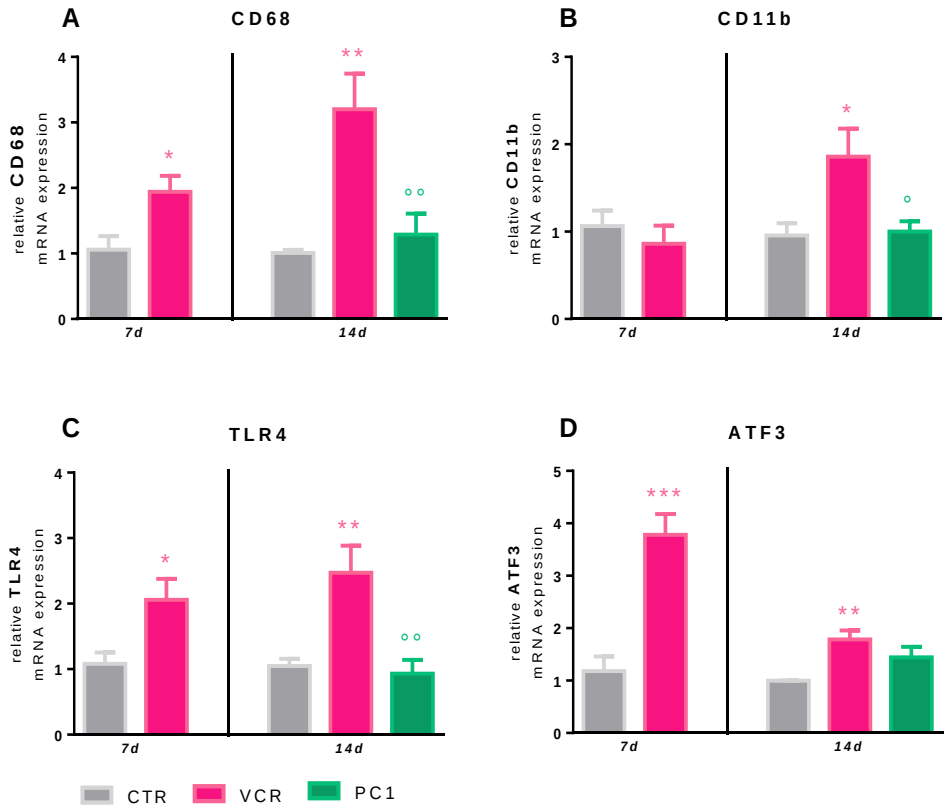


Figure 14 | Neuroimmune activation in dorsal root ganglia.
Seven days after the first VCR injection (VCR day 7) and at the end of VCR/VCR+PC1 protocol (VCR day 14), mRNA levels of the neuroimmune markers CD68 (panel A), CD11b (panel B), TLR4 (panel C), and of the marker of cell stress/ tissue damage ATF3 (panel D) were measured in DRG. Expression levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data represent mean±SEM of 5-8 mice per group. At day 7 statistical analysis was performed by means of unpaired T- test while at day 14 by means of One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR (at the same time point); °p<0.05, °°p<0.01 vs VCR day 14.

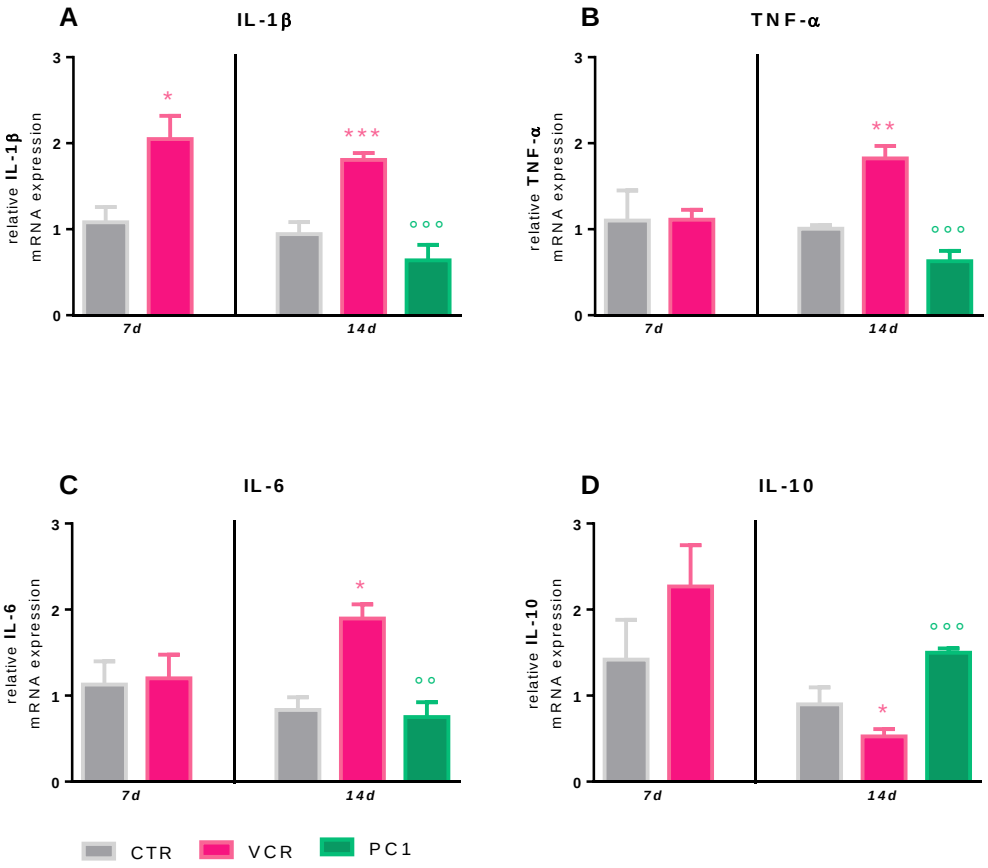


Figure 15 | Cytokine expression levels in dorsal root ganglia.
Panels A, B and C represent respectively mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6; panel D reports those of the anti-inflammatory cytokine IL-10. Analysis were performed 7 days after the first VCR administration (7d), before starting PC1 treatment (CTR and VCR groups), and at the end VCR/ VCR+ PC1 protocol (14d, CTR, VCR, VCR+ PC1 groups). mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 5-8 mice per group. At day 7 statistical analysis was performed by means of unpaired T- test while at day 14 by means of One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR (at the same time point); °°p<0.01, °°°p<0.001 vs VCR day 14.

As illustrated in **figure 14**, at day 7 DRG of VCR-mice showed an increase of CD68 mRNA levels (**panel A**). Simultaneously (day 7), we also observed increased expression TLR4 (**panel C**) and of the marker of cell stress/ injury ATF3 (**panel D**). At the end of the VCR schedule (day 14), mice treated with the antineoplastic drug still showed an upregulation of CD68 (**panel A**), TLR4 (**panel C**), ATF3 (**panel D**) but at this time point also CD11b, that was not altered at VCR day 7, resulted upregulated (**panel B**).
As it happened in BTZ-mice also in animals treated with VCR, PC1 chronic treatment was able to contrast macrophage recruitment and activation, as demonstrated by the reduction of CD68 (**panels A**), CD11b (**panel B**) and TLR4 expression (**panel C**), but it was unable to contrast the neuronal damage, as demonstrated by ATF3 expression levels (**panel D**).

As shown in **figure 15** while at day 7 only a slight increase of IL-1β (**panel A**) and no alteration in TNF-α, IL-6 and IL-10 (**panels B**,**C** and **D**) was present in the DRG of VCR-mice, at the end of VCR protocol (day 14), this nervous tissue showed a considerable neuroinflammatory condition characterized by a marked pro-inflammatory cytokine profile due to high levels of IL-1β (**panel A**), TNF-α (**panel B**) and IL-6 (**panel C**) and low levels of the anti-inflammatory cytokine IL-10 (**panel D**).
PC1 treatment was able to counteract these neuroinflammatory alterations, restoring a correct cytokine balance: reducing IL-1β (**panel A**), preventing TNF-α and IL-6 increase (**panels B** and **C**) and opposing to IL-10 decrease (**panel D**).

6.2.3 Prokineticin system activation in the spinal cord

Similarly to DRG, also in spinal cord (**figure 16**) at VCR day 7 we only observed increased mRNA levels of both PK-Rs (**panels B** and **C**) without changes of PK2 (**panel A**). At the end of VCR protocol (day 14), in the same tissue, an upregulation of the entire PK system (**panels A**, **B** and **C**) was evident. PC1 treatment was able to normalize expression of all PK system members, preventing PK2 upregulation (**panel A**) and reducing PK-Rs levels (**panels B** and **C**).

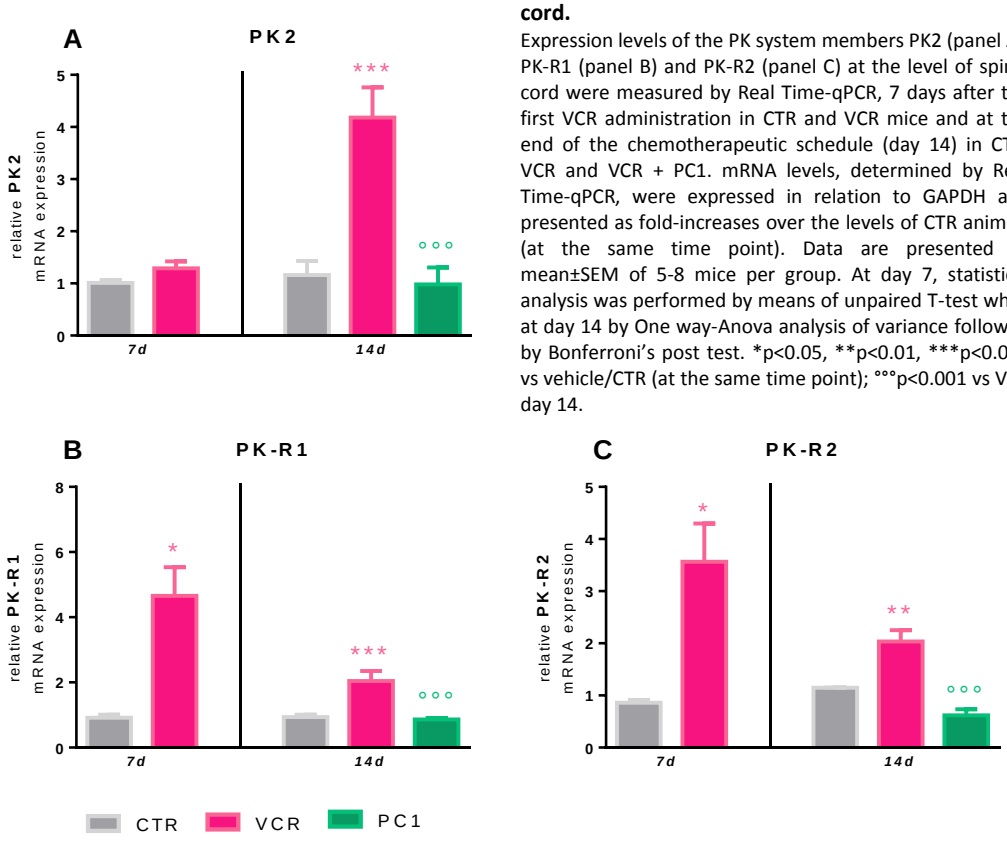


Figure 16 | Prokineticin system activation in spinal cord.
Expression levels of the PK system members PK2 (panel A), PK-R1 (panel B) and PK-R2 (panel C) at the level of spinal cord were measured by Real Time-qPCR, 7 days after the first VCR administration in CTR and VCR mice and at the end of the chemotherapeutic schedule (day 14) in CTR, VCR and VCR + PC1. mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 5-8 mice per group. At day 7, statistical analysis was performed by means of unpaired T-test while at day 14 by One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR (at the same time point); °°°p<0.001 vs VCR day 14.

6.2.4 Effect of PK-Rs antagonism on spinal cord neuroinflammation

As shown in **figure 17**, at the first analyzed time point (VCR day 7) in the central nervous system a significant upregulation of CD68 (**panel A**) without any alteration in CD11b (**panel B**) TLR4 (**panel C**), astrocytes marker GFAP (**panel D**) and ATF3 (**panel E**) expression was present. Differently, at the end of VCR treatment (VCR day 14), we detected increased levels of CD68 (**panel A**) but also CD11b (**panel B**), TLR4 (**panel C**), GFAP (**panel D**) and ATF3 (**panel E**).

Also in this nervous tissue PC1 chronic administration was able not only to reduce the neuroimmune activation, as demonstrated by the decreased expression of CD68 (**panel A**), CD11b (**panel B**), TLR4 (**panel C**) but also to contrast cell stress/tissue damage (**panel E**). However, the PK-Rs antagonist was unable to contrast the upregulation of the astrocytes marker GFAP, that was observed at the higher VCR cumulative injected dose (**panel D**).

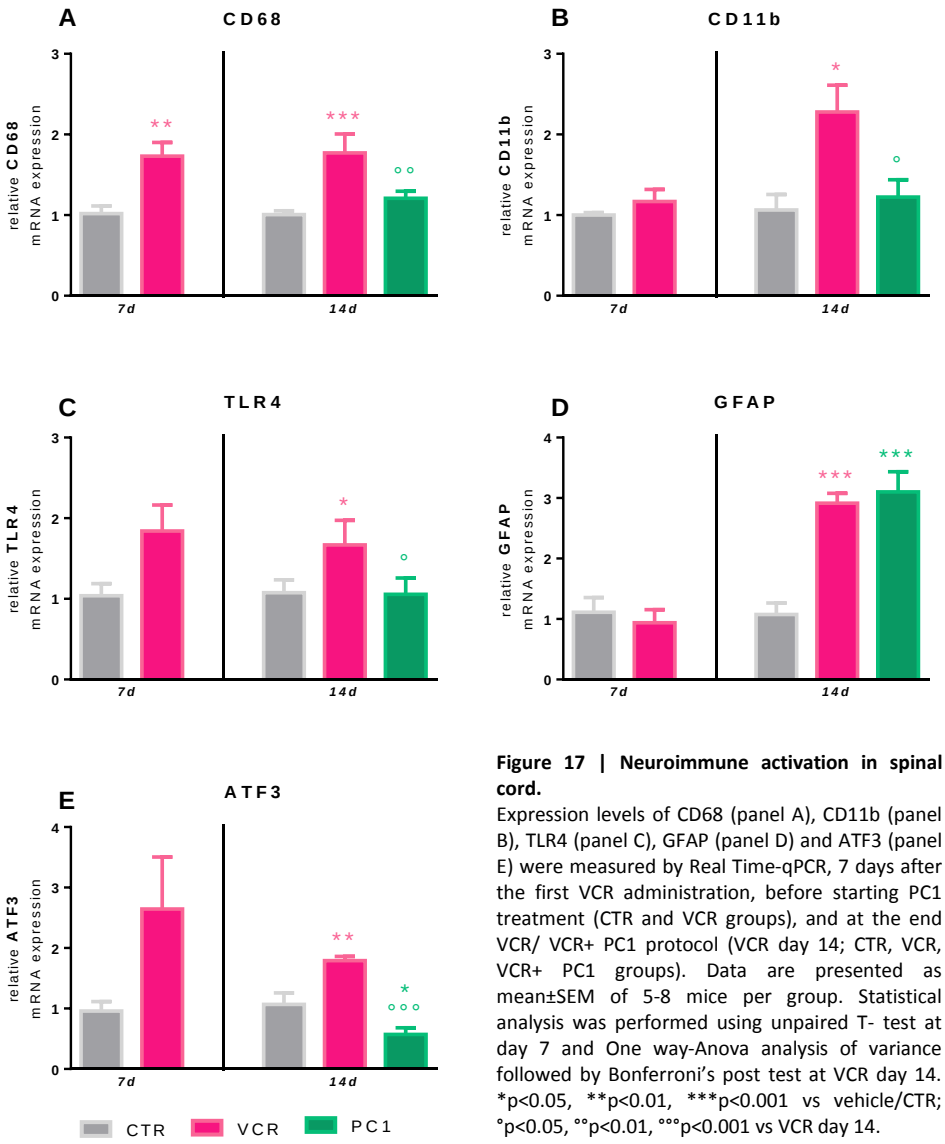


Figure 17 | Neuroimmune activation in spinal cord.
Expression levels of CD68 (panel A), CD11b (panel B), TLR4 (panel C), GFAP (panel D) and ATF3 (panel E) were measured by Real Time-qPCR, 7 days after the first VCR administration, before starting PC1 treatment (CTR and VCR groups), and at the end VCR/ VCR+ PC1 protocol (VCR day 14; CTR, VCR, VCR+ PC1 groups). Data are presented as mean±SEM of 5-8 mice per group. Statistical analysis was performed using unpaired T- test at day 7 and One way-Anova analysis of variance followed by Bonferroni's post test at VCR day 14. *p<0.05, **p<0.01, ***p<0.001 vs vehicle/CTR; °p<0.05, °°p<0.01, °°°p<0.001 vs VCR day 14.

In accordance with what observed so far, also in this case spinal cord seemed to be less affected by chemotherapy neurotoxicity than DRG, in fact, as shown in **figure 18**, 7 consecutive days of VCR administration did not induce any change in the mRNA levels of IL-1β (**panel A**), TNF-α (**panel B**), IL-6 (**panel C**) and IL-10 (**panel D**) at the level of this nervous tissue. Differently, at the end of the chemotherapeutic

schedule (VCR day 14) we observed an upregulation of the pro-inflammatory cytokines IL-1β (**panel A**) and TNF-α (**panel B**) in VCR-mice. PK-Rs antagonist chronic administration was able to prevent this switch toward a pro-inflammatory profile development in the spinal cord (**panels A** and **B**).

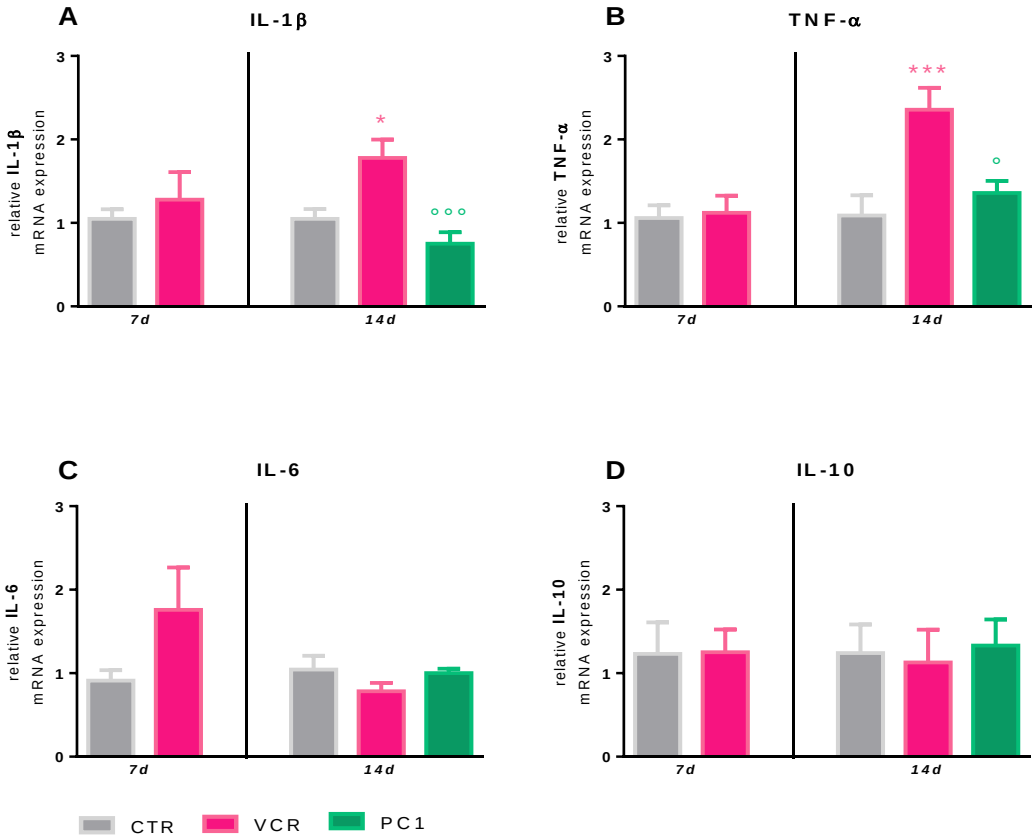


Figure 18 | Cytokine expression levels in spinal cord.
Panels A, B and C represent respectively mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6; panel D reports those of the anti-inflammatory cytokine IL-10. Analysis were performed 7 days after the first VCR administration, before starting PC1 treatment (CTR and VCR groups), and at the end VCR/ VCR+ PC1 protocol (VCR day 14; CTR, VCR, VCR+ PC1 groups). mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals (at the same time point). Data are presented as mean±SEM of 5-8 mice per group. At day 7 statistical analysis was performed by means of unpaired T- test while at day 14 by means of One way-Anova analysis of variance followed by Bonferroni's post test. *p<0.05 vs vehicle/CTR (at the same time point), ***p<0.001; °p<0.05, °°°p<0.001 vs VCR day 14.

6.2.5 Effect of PK-Rs antagonism on multiple vincristine cycles

As shown in **Figure 19**, following the interruption of VCR schedule of the duration of 14 days, hypersensitivity was monitored over time by measuring mechanical allodynia. One week after VCR interruption (day 21), VCR-mice started to show a slight trend toward recover; in PC1-mice the antagonist's effect was still evident.

Six weeks after VCR interruption (day 65) mice belonging to both experimental groups (VCR and VCR+PC1) completely recovered as demonstrated by their paw withdrawal thresholds that were equivalent to those of control mice.

Also in this experimental protocol, as we did in the BTZ experiments, at day 80, we started to administer to animals previously treated with the chemotherapeutic agent a new VCR schedule using the same protocol of the first VCR cycle (i.p., 0.1mg/kg daily for 14 consecutive days).

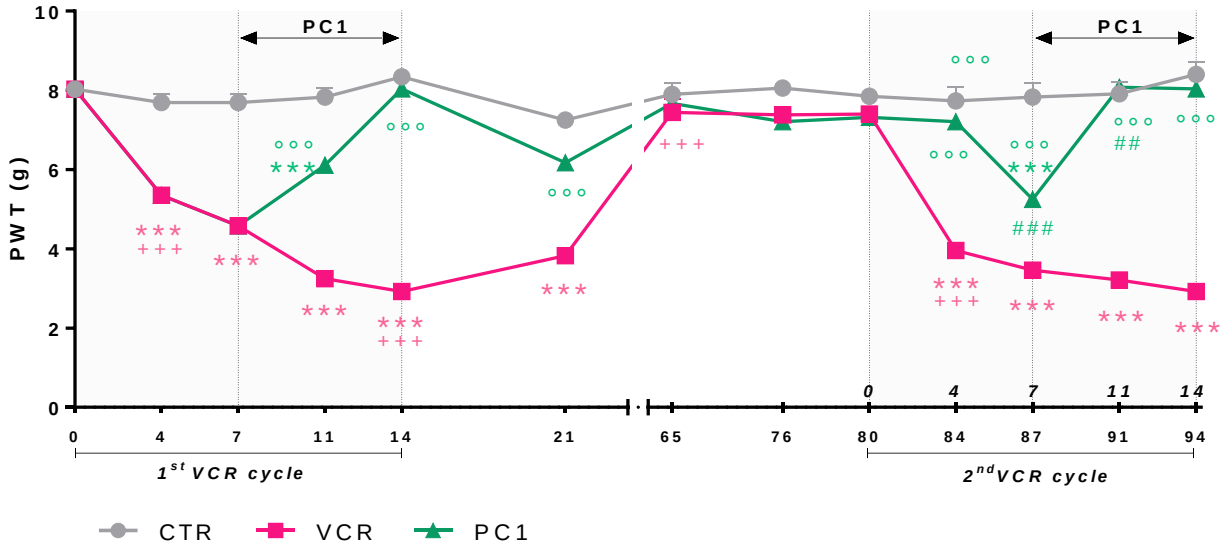


Figure 19 | Mechanical allodynia development during a second VCR cycle.
Following the interruption of the chemotherapeutic protocol of 14 days (VCR 0.1 mg/kg once a day for 14 consecutive days, i.p.) and PC1 (s.c. 150 µg/kg twice day) chronic treatment (from VCR day 7 to 14), mice progressively recovered from hypersensitivity. At day 80, in the presence of basal mechanical thresholds, mice that had been previously treated with the chemotherapeutic drug (first VCR cycle) underwent a second treatment with vincristine, using the same experimental protocol (VCR, i.p., 0.1 mg/kg, once a day for 14 consecutive days). At day 87, mice previously treated with PC1 started a new chronic treatment with the antagonist (s.c. 150 µg/kg twice day). All animals were monitored until the end of the second VCR and PC1 treatment (14 days since the beginning of the second cycle corresponding to 94 days after the first VCR injection). Data represent mean ± SEM of 6 mice/group. Statistical analysis was performed by means of Two way-ANOVA followed by Bonferroni's post-test. ***p <0.001 vs vehicle/CTR; °°°p<0.001 vs VCR at the same time point; $^{+++}$p<0.001 vs the previous VCR dose; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs VCR at the same time point during the 1st VCR cycle.

The second VCR cycle induced again an allodynic effect that was similar to the one observed during the first VCR schedule, but interestingly, at day 84 (that is 4 days after the beginning of the second VCR schedule), in mice that had been previously treated with PC1, the allodynic effect induced by VCR was less intense in comparison with that observed in VCR-only re-treated mice. At day 87 (7 days after the beginning of the second VCR schedule) also VCR+PC1 mice developed hypersensitivity even if their PWT were still significantly different from those of VCR-mice. At this time (day 87), mice previously treated with the antagonist started a second PC1 chronic treatment (s.c., 150µg/kg twice a day/7 consecutive days). PK-Rs antagonist was able to completely reverse mechanical allodynia as demonstrated by the PWT of PC1-treated mice that, at the end of the VCR/PC1 schedule (day 94), were equivalent to those of controls. Interestingly, the positive effect induced by the second PC1 chronic treatment was faster (day 91, that is 11 days after the beginning of the second PC1 treatment) than the one observed during the first cycle (day 14), suggesting a protective role of the PK-Rs antagonist in the development of the painful symptomatology induced by a second vincristine cycle.

6.3 *In vitro* evaluations

Most chemotherapeutic agents do not penetrate the blood-brain barrier (BBB) but they can cross the blood nerve barrier (BNB) and they accumulate in dorsal root ganglia and peripheral nerves. Peripheral sensory neurons in DRG and their axons are particularly susceptible to the collateral damage due to chemotherapy. Sensory neurons vulnerability derives from multiple factors including the lack of an efficient neurovascular barrier, allowing unconstrained drug permeation, and by the presence of a dense vascularisation and fenestrated capillaries of DRG which make neurons further exposed to the toxic drugs (Lees et al., 2017; Montague and Malcangio 2017).

Therefore, we decided to begin to investigate the neuronal contribution to CIPN development and the possible effect of the PK-Rs antagonist on neurons, by using an *in vitro* model represented by primary sensory neurons collected from lumbar DRG of wildtype healthy mice. Since neurons are able of reproducing *in vitro* the basic architectural rearrangements that are observed *in vivo*, this model allowed us to better understand the morphological changes related to BTZ or VCR exposure.

6.3.1 Neurite outgrowth of DRG primary sensory neurons exposed to bortezomib

In **figure 20** are shown the results of the neurite outgrowth assessed in DRG primary sensory neurons treated *in vitro* with four different nanomolar doses (4, 6, 8 and 10nM) of bortezomib.

As shown in **panel A**, **picture b**, cells exposed for 72h to the lower BTZ concentration (4nM) did not show any alteration in the total neurite length. However, by increasing the concentration of the chemotherapeutic drug (BTZ 6nM) we observed a significant decrease of neurite length (**panel A** and **picture c**) and higher BTZ concentrations (8 and 10 nM) strongly altered neuronal arborisation (**panel A, pictures d** and **e**).

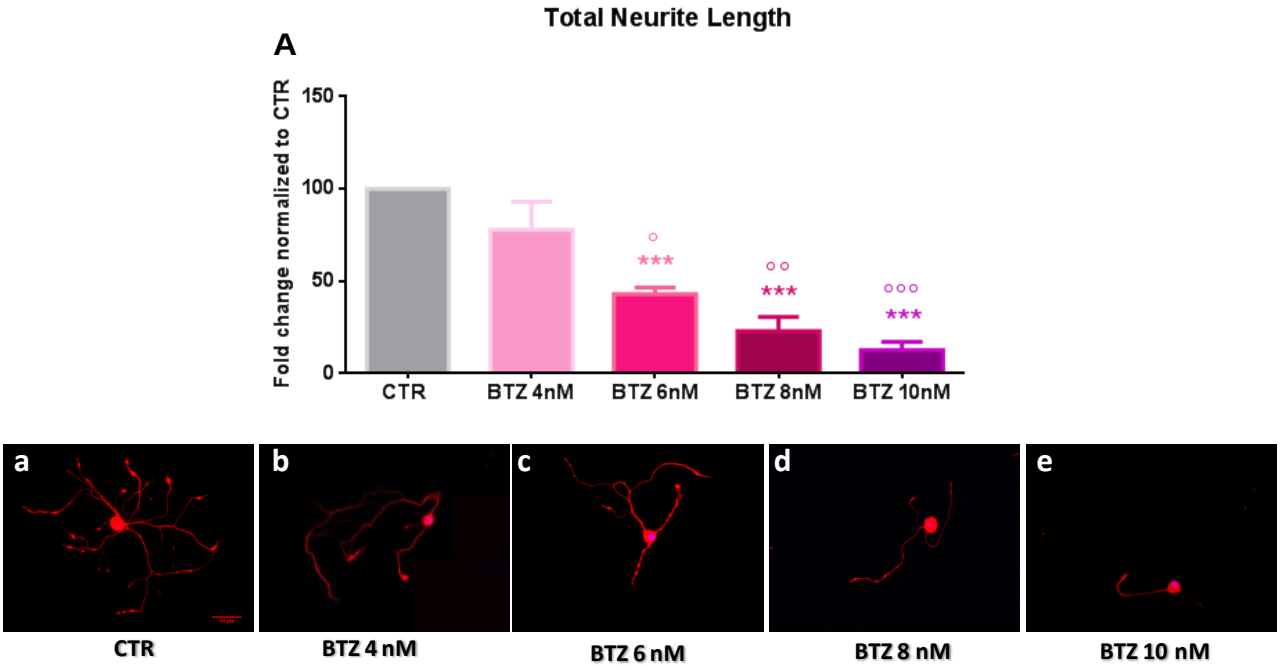


Figure 20 | Neurite outgrowth of DRG primary sensory neurons exposed to BTZ.
Neurite outgrowth assay of DRG primary sensory neurons collected from wildtype healthy mice and treated *in vitro* with the vehicle (culture medium, CTR, picture a) or with the following BTZ concentrations: 4, 6, 8 and 10nM. Representative images (a-e) of β3-tubulin (Tuj1; red) staining in DRG cell cultures. Cell nuclei were countrastained with DAPI (blue). Scale bar=50μM. Data are presented as fold change increase over the levels of CTR condition. Mean±SEM, N=4, n≥80; One way-ANOVA followed by Bonferroni's post-test. ***p<0.001 vs CTR; °p<0.05, °°p<0.01, °°°p<0.001 vs BTZ 4nM.

6.3.2 Effect of PK-Rs antagonist on neurite outgrowth alterations due to bortezomib exposure

Since BTZ 6nM induced about 50% reduction in neurite length, this concentration was chosen to investigate the possible involvement of the Prokineticin system by analysing the *in vitro* effect of the PK-Rs antagonist . Different concentrations of PC1 were co-applied with bortezomib 6nM and cells were incubated for 72h.
As shown in **figure 21** PC1 dose-dependently prevented BTZ-induced reduction of neurite length, in fact already at the lower doses, PC1 50 and 100nM (**pictures c** and **d**), we detected a slight increase of neurite length compared to cells exposed only to BTZ. Increasing the concentration of the antagonist, 250 and 500 nM (**pictures e** and **f**), a stronger improvement of neuronal arborization was observed. However, when PC1 concentration reached 1µM (**pictures g**) no benefic effect was detected.
We also tested all PC1 concentrations in the absence of the chemotherapeutic agent in order to investigate possible effects of the PK-Rs antagonist alone on the neuronal morphology. No differences were detected among PC1 50, 100, 250, 500 nM and vehicle-treated neurons. On the other hand, PC1 1µM alone induced a significant reduction of neurite length.

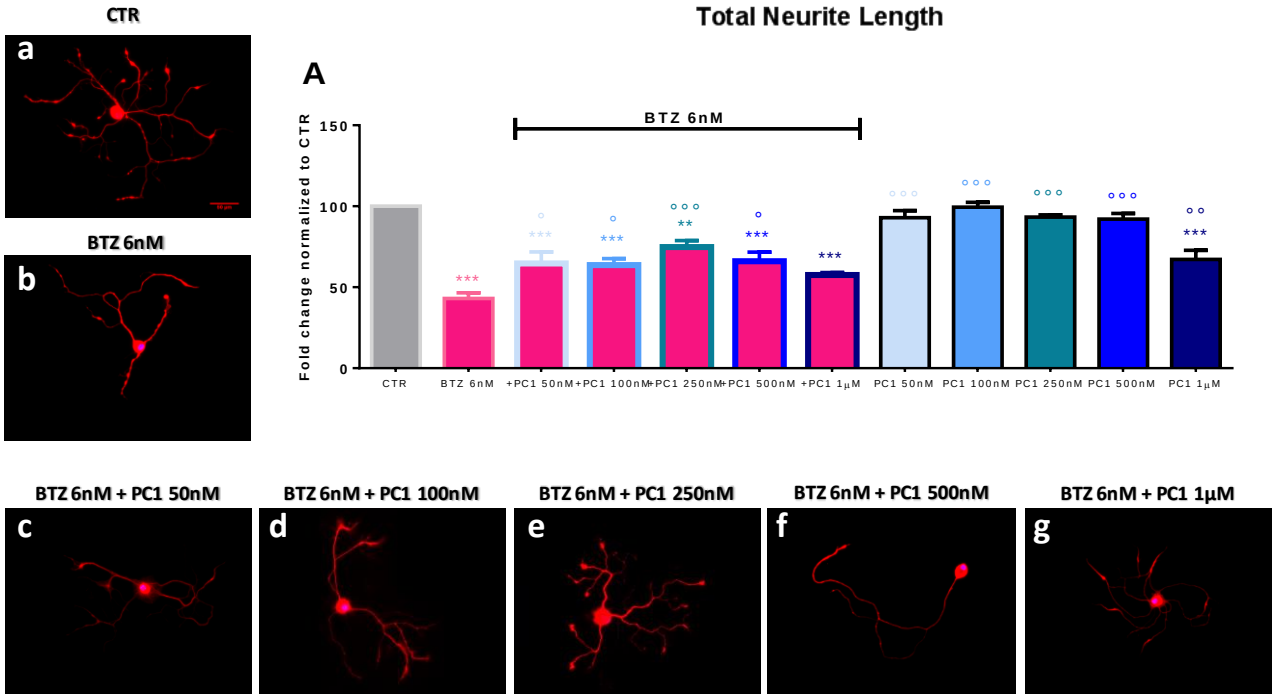


Figure 21 | Neurite outgrowth of DRG primary sensory neurons exposed to BTZ+PC1.
Effect of different doses (50nM, 100nM, 250nM, 500nM and 1µM) of the PK-Rs antagonist PC1 on DRG primary sensory neurons outgrowth, when the PK-Rs antagonist was *in vitro* administered alone or in association with BTZ 6nM. Representative images (a-g) of β3-tubulin (Tuj1; red) staining in DRG cell cultures. Cell nuclei were countrastained with DAPI (blue). Scale bar=50µM. Data are presented as fold change increase over the levels of CTR condition. Mean±SEM, N=4 n≥80; One way-ANOVA followed by Bonferroni's post-test. **p<0.01, ***p<0.001 vs CTR; °p<0.05, °°p<0.01, °°°p<0.001 vs BTZ 6nM.

6.3.3 Neurite outgrowth of DRG primary sensory neurons exposed to vincristine

Total neurite length was measured also in cell cultures exposed to different doses of vincristine.
Since from the literature the majority of the studies evaluating the *in vitro* effect of vincristine were performed using brain neurons and considering the heterogeneity of VCR doses and/or timing of incubation, we decided to test a wider range of concentrations: from 0.01nM to 100µM.
As shown in **figure 22 panel A**, vincristine had a cytotoxic action on neurons in a dose dependent manner; cells expose to the lower tested doses, 0.01nM (**picture b**) and 0.1nM (**picture c**), already showed a significant decrease in the total neurite length. From VCR 1nM (**picture d**) we observed a further reduction

of neuronal processes which was similar also for VCR 5nM (**picture e**) and 10nM (**picture f**) and it was then emphasized for VCR 50nM (**picture g**), 100nM (**picture h**), 1µM (**picture i**) and 5µM (**picture l**). Finally, cells incubated with VCR 10µM (**picture m**), 20 µM (**picture n**), 50 µM (**picture o**) and 100 µM (**picture p**) did not show neurites.

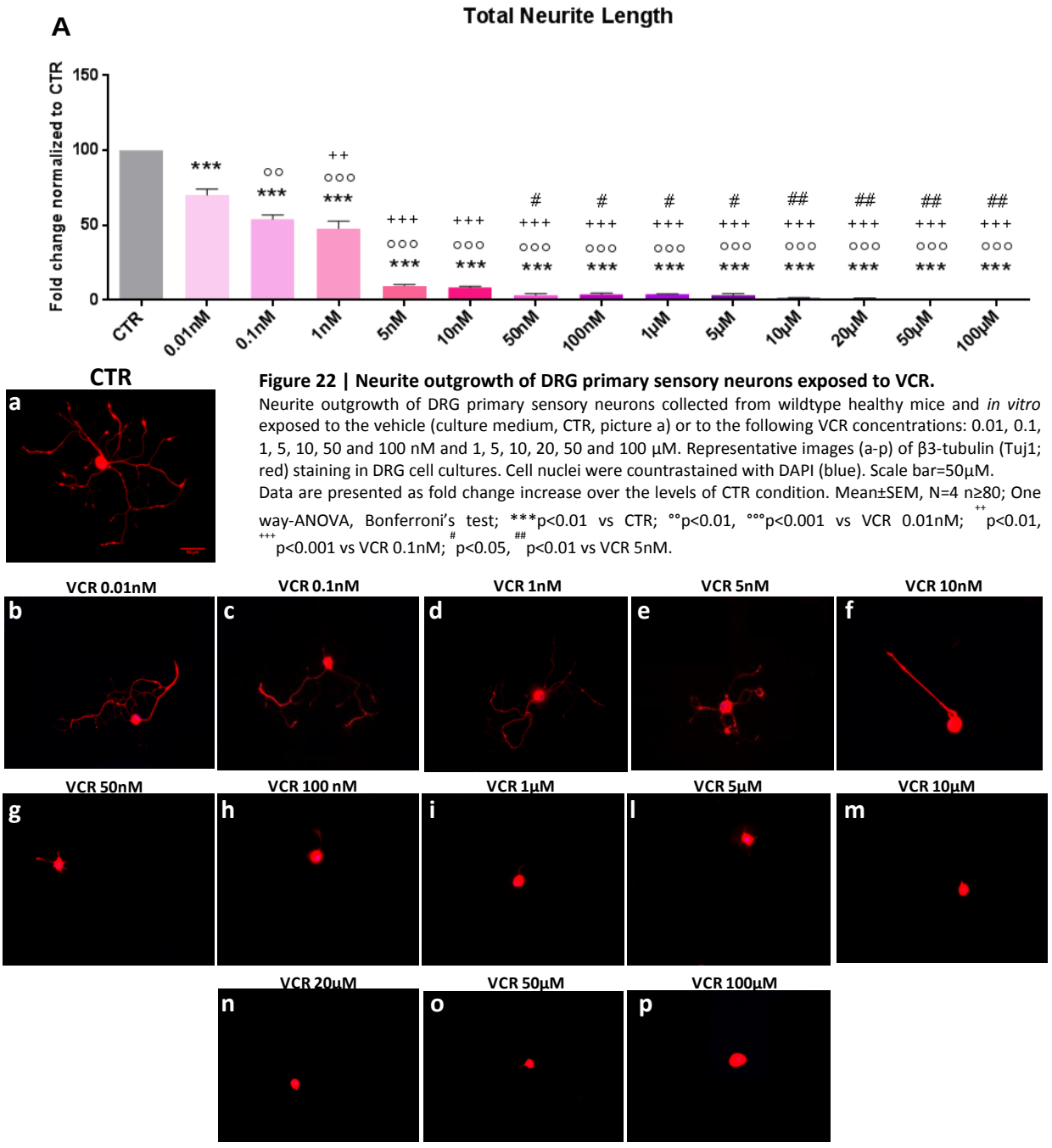


Figure 22 | Neurite outgrowth of DRG primary sensory neurons exposed to VCR.
Neurite outgrowth of DRG primary sensory neurons collected from wildtype healthy mice and *in vitro* exposed to the vehicle (culture medium, CTR, picture a) or to the following VCR concentrations: 0.01, 0.1, 1, 5, 10, 50 and 100 nM and 1, 5, 10, 20, 50 and 100 µM. Representative images (a-p) of β3-tubulin (Tuj1; red) staining in DRG cell cultures. Cell nuclei were countrastained with DAPI (blue). Scale bar=50µM.
Data are presented as fold change increase over the levels of CTR condition. Mean±SEM, N=4 n≥80; One way-ANOVA, Bonferroni's test; ***p<0.01 vs CTR; °°p<0.01, °°°p<0.001 vs VCR 0.01nM; $^{++}$p<0.01, $^{+++}$p<0.001 vs VCR 0.1nM; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs VCR 5nM.

6.3.4 Effect of PK-Rs antagonist on neurite outgrowth alterations due to vincristine exposure

Given the results obtained in the *in vitro* bortezomib experiments, we decided to test whether the PK-Rs antagonist could be able to contrast also vincristine toxicity.

PC1 250nM was the most efficacious dose in preventing neurite length reduction in BTZ experiments, therefore this concentration was used also in this set of experiments. PK-Rs antagonist effect was tested on concentrations of VCR that were chosen from the previous data (**figure 22**) because they were able to induce a significant reduction of neurite outgrowth when compared not only to vehicle-treated cells but also one versus the other: 0.01nM, 0.1nM, 1nM and 50nM.

As shown in **figure 23**, PC1 was effective in contrasting the morphological alterations induced by VCR 0.01nM (**pictures c** and **d**), 0.1nM (**picture e** and **f**) and 1nM (**picture g** and **h**). No positive effect of PC1 250nM was observed in neurons simultaneously exposed to it and VCR 50nM (**picture i** and **l**). PC1 250nM did not induce any alterations in neurite outgrowth in comparison to CTR (**pictures b** and **a**, respectively).

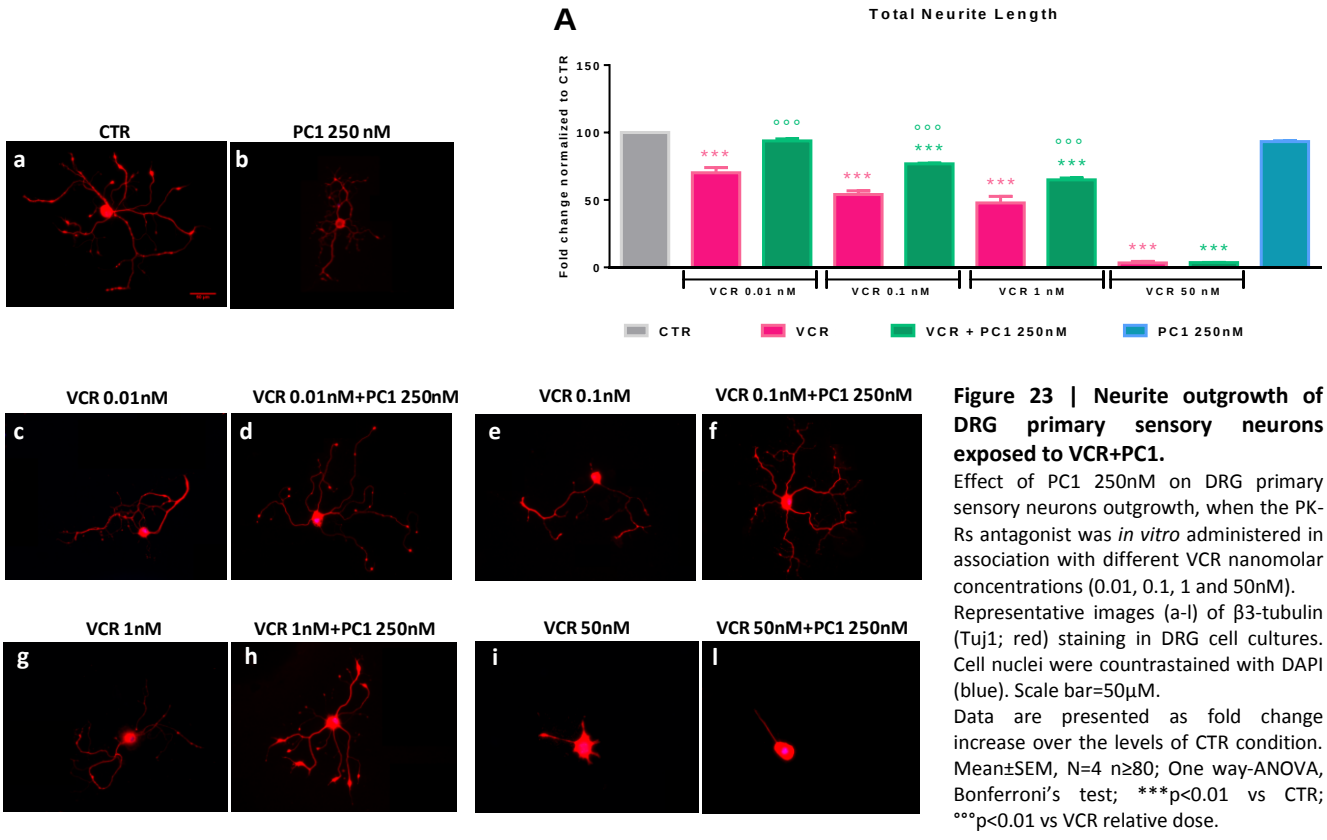


Figure 23 | Neurite outgrowth of DRG primary sensory neurons exposed to VCR+PC1.
Effect of PC1 250nM on DRG primary sensory neurons outgrowth, when the PK-Rs antagonist was *in vitro* administered in association with different VCR nanomolar concentrations (0.01, 0.1, 1 and 50nM).
Representative images (a-l) of β3-tubulin (Tuj1; red) staining in DRG cell cultures. Cell nuclei were countrastained with DAPI (blue). Scale bar=50μM.
Data are presented as fold change increase over the levels of CTR condition. Mean±SEM, N=4 n≥80; One way-ANOVA, Bonferroni's test; ***p<0.01 vs CTR; °°°p<0.01 vs VCR relative dose.

6.3.5 Prokineticin system activation in DRG cell cultures

Given the results obtained in the neurite outgrowth assay, and considering the higher toxicity of vincristine if compared to bortezomib, we decided to better investigate the role of prokineticin system in the neuronal alterations induced by vincristine.

In order to localize PK2 in our cell cultures (**figure 24**) we performed an immunofluorescent staining of Tuj1 (**panel A - pictures a, d** and **g**) and PK2 (**pictures b, e** and **h**) in control cells. PK2, in vehicle-treated cells seemed to be localized both on neurons (**panel A - pictures c** and **f**) and in other cell types present in the cultures (**panel A - picture i**), which could probably be satellite cells.

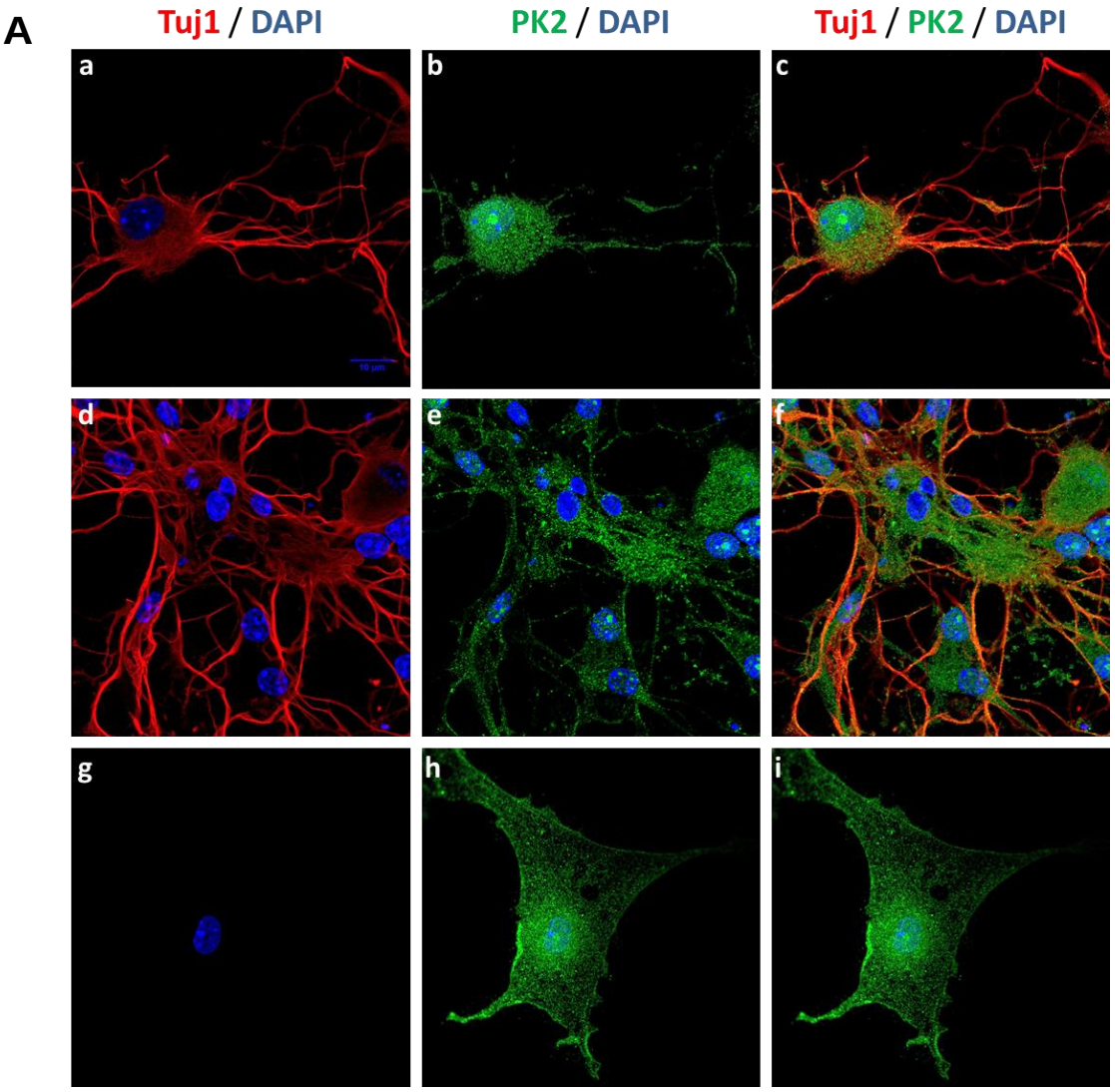
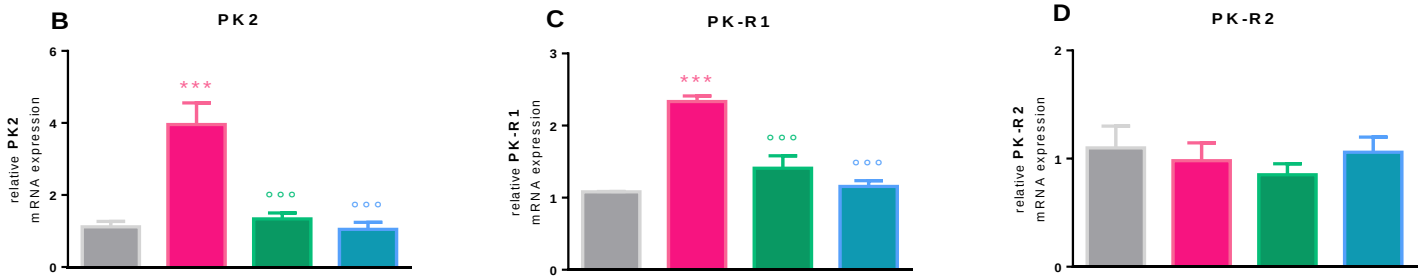


Figure 24 | Prokineticin system activation in VCR treated DRG cell cultures.
Panel A (pictures a-i) shows representative images of β3-tubulin (Tuj1; red) and PK2 (green) signal in vehicle-treated DRG cell cultures. Cell nuclei were countrastained with DAPI (blue). Scale bar= 10µm. N=4.
Expression levels of PK2 (panel B), PK-R1 (panel C), PK-R2 (panel D) were measured in DRG cell cultures treated with vehicle (CTR), VCR 1nM alone or in association with PC1 250nM (VCR+PC1) and PC1 250nM without the chemotherapeutic agent. mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR condition. Data are presented as mean±SEM from 10 mice per condition. Statistical analysis was performed by mean of One Way-ANOVA followed by Bonferroni's post-test. ***p<0.001 vs CTR; °°°p<0.001vs VCR 1nM.



In the same figure is shown how cells exposed to vincristine 1nM displayed an upregulation of PK2 (**panel B**) and PK-R1 (**panel C**) mRNA levels while no differences were detected in PK-R2 (**panel D**). When cells were simultaneously incubated with PC1 250nM and VCR 1nM, both PK2 (**panel B**) and PK-R1 (**panel C**) upregulation was prevented.
PK-Rs antagonist alone, at the dose of 250nM, did not induce any change in the expression levels of the PK system components (**panels B, C** and **D**).

6.3.6 Neuroimmune markers expression in DRG cell cultures

Since from our preliminary immunohistochemical data on PK2 localization it seemed to be present also in non-neuronal cells, we measured expression levels of GFAP, marker of satellite cells, in our cultures. As shown in **figure 25 panel A**, no significant difference was detected in the GFAP mRNA between cells exposed to VCR 1nM and the controls.

However, expression of TLR4 (**panel B**) and of the pro-inflammatory cytokines IL-1β (**panel C**), IL-6 (**panel E**) and of the anti-inflammatory cytokine IL-10 (**panel F**) in the VCR1nM-cells resulted upregulated, as well as ATF3 (**panel G**). No changes were present in TNF-α mRNA (**panel D**). Interestingly, these alterations were prevented when cells were simultaneously incubated with VCR 1nM and PC1 250 nM, as shown in **panels B**, **C**, **E**, **F** and **G**. PC1 250nM alone did not induce any change in the expression of GFAP (**panel A**), TLR4 (**panel B**), pro- (**panels C**, **D** and **E**) and anti- inflammatory cytokines (**panel F**) and ATF3 (**panel G**).

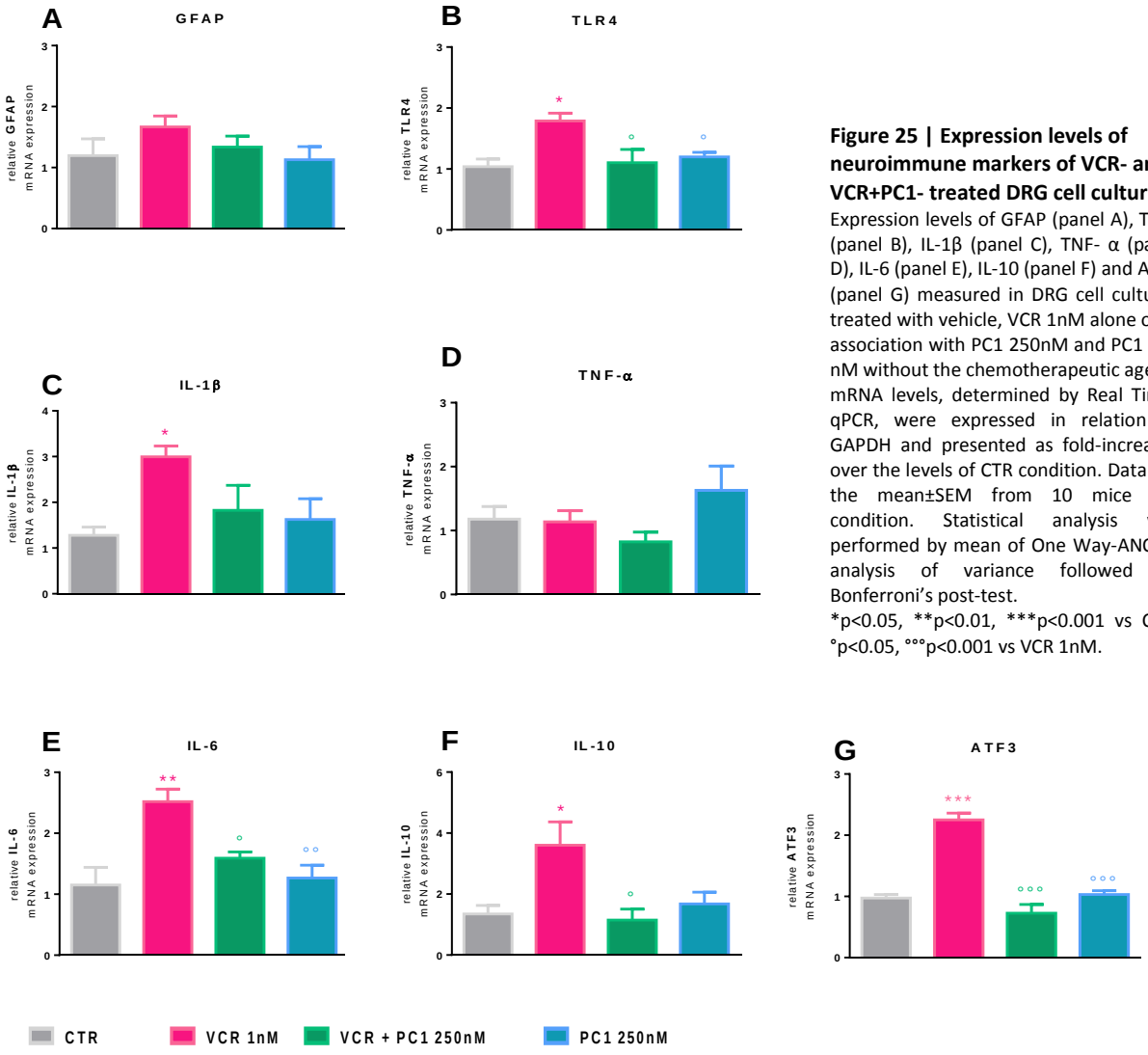


Figure 25 | Expression levels of neuroimmune markers of VCR- and VCR+PC1- treated DRG cell cultures. Expression levels of GFAP (panel A), TLR4 (panel B), IL-1β (panel C), TNF- α (panel D), IL-6 (panel E), IL-10 (panel F) and ATF3 (panel G) measured in DRG cell cultures treated with vehicle, VCR 1nM alone or in association with PC1 250nM and PC1 250 nM without the chemotherapeutic agent. mRNA levels, determined by Real Time-qPCR, were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR condition. Data are the mean±SEM from 10 mice per condition. Statistical analysis was performed by mean of One Way-ANOVA analysis of variance followed by Bonferroni's post-test.
*p<0.05, **p<0.01, ***p<0.001 vs CTR; °p<0.05, °°°p<0.001 vs VCR 1nM.

7. Discussion

In the present work we provide evidences about the involvement of the chemokine-like protein prokineticin 2 (PK2) and its receptors, PK-R1 and PK-R2, in the onset and chronicization of experimental chemotherapy-induced peripheral painful neuropathy, one of the main dose-limiting side effects of antineoplastic therapy. Furthermore, we demonstrated a protective role of the PK-Rs antagonist PC1 on the pathology progression.

The involvement of PK system was assessed using a mouse model of CIPN, induced by the administration of two different chemotherapeutic agents commonly used in clinics and related to the development of CIPN: vincristine (VCR), the most neurotoxic drug among Vinca Alkaloids, and bortezomib (BTZ), the first of a new class of agents that inhibits the 26s complex of proteasome favouring apoptosis of cancer cells. These drugs, despite having completely different mechanisms of action on tumor cells (Fehrenbacher 2019) , are known to induce neurotoxicity and consequent neuropathy with similar common mechanisms (Flatters et al., 2017; Boyette-Davis et al., 2015).

In our protocols, CIPN was induced in mice by using a chemotherapeutic agents dosage able to induce a detectable neuropathic phenotype but at the same time limiting systemic side effects (Boehmerle et al., 2014; Kiguchi et al., 2008). As it happens in the majority of CIPN experimental models, we use the administration of the chemotherapeutic agent without tumour load: this is due both to ethical reasons and practical doability. In fact, pain-like behaviours cannot be accurately assessed if animals are ill as a result of cancer systemic toxicity and therefore lethargic/unresponsive to hind paw stimulation. However, as in clinics chemotherapy is often received after surgical removal of the tumour in order to remove possible micro-metastases, modelling CIPN through chemotherapy administration alone can be considered an effective approach (Flatters et al., 2017).

In our experiments, both bortezomib and vincristine induced in mice a painful neuropathy characterized by the presence of mechanical and thermal allodynia as well as thermal hyperalgesia, which are among the main symptoms usually referred by patients (Boyette-Davis et al., 2018). The involvement of the PK system in CIPN was clearly supported by the results obtained in the behavioural tests. In fact, blocking PK2/PK-Rs signaling through the use of a PK-Rs antagonist, PC1, was effective in controlling painful symptomatology, completely reverting thermal allodynia and significantly ameliorating mechanical allodynia and thermal hyperalgesia.

The results obtained in the behavioural tests well correlated with the observed neuroinflammatory alterations in the peripheral and central nervous stations of pain transmission. The involvement of neuroimmune components in the etiopathogenesis of CIPN has been widely studied and several preclinical data have demonstrated that antineoplastic drugs are able to activate both innate and adaptive immune responses in peripheral and central nervous system (Starobova and Vetter, 2017; Qu et al., 2012). In particular, since chemotherapeutics can cross the blood-nerve barrier, they accumulate in dorsal root ganglia and in the peripheral nerves where they exert a toxic action which induces a further immune cell infiltration and activation (Montague and Malcangio, 2017).

To assess specifically the role of PK system in our models, for each experimental protocol (BTZ and VCR) we performed biochemical analysis at two different time points: before starting PC1 treatment (day 14 for BTZ and day 7 for VCR), and at the end of chemotherapeutic schedule (BTZ day 28 and VCR day 14).

Consistently with what above mentioned, our biochemical results indicate that already after 14 days of BTZ, in the presence of an hyperalgesic and allodynic state, peripheral nervous tissues (i.e. sciatic nerve and DRG) were characterized by increased levels of macrophage activation markers, i.e. CD68 and TLR4, and by the presence of a pro-inflammatory cytokine profile due to high levels of TNF-α, IL-1β, and IL-6 and low levels of the anti-inflammatory cytokine IL-10, which was particularly evident in DRG.

A similar macrophage activation was observed in the DRG of VCR-treated mice at day 7, as demonstrated by the increased CD68 and TLR4 expression levels. However, in VCR-mice the pro-inflammatory profile was less pronounced with only an upregulation of the pro-inflammatory cytokine IL-1β.

According with what already observed in studies with other chemotherapeutics (Peters et al., 2007; Cavaletti et al., 1995; Warwick et al., 2013), in the peripheral nervous system of BTZ- and VCR- mice, we detected an upregulation of Activating Transcription Factor 3 (ATF3), an adaptive responsive gene, which is considered a marker of cell injury. ATF3 increased expression levels could be due not only to alterations in neurons but also to Schwann and Satelite cells that respond to chemotherapy by undergoing phenotypic changes and stimulating the secretion of mediators that enhance neuronal excitability and may worsen painful symptomatology (Lees et al., 2017).

At this precocious time point (BTZ day 14 and VCR day 7), the central nervous system did not seem to be directly affected by chemotherapy toxicity, according to the fact that these kind of drugs do not or just in small part cross the blood brain barrier (Carozzi et al., 2014; Han and Smith 2013).

In fact, spinal cord of BTZ mice at day 14 only showed an increase of the astrocytes marker GFAP without any other biochemical alteration. Differently, in VCR-mice at day 7, we only detected a microglia alteration, as shown by the CD68 upregulation and no other change. The reasons behind this differential cell activation has not been studied yet, but we would like to deeply investigate this aspect in our future experiments.

In our study, at the end of the chemotherapeutic schedule (BTZ day 28 and VCR day 14), we observed a further lowering of the response thresholds to mechanical stimuli in mice. The increased hypersensitivity correlated with the appearance of a stronger neuroinflammatory profile, as demonstrated by the increased expression levels of pro-inflammatory cytokines, in the analyzed peripheral nervous tissue of mice undergoing chemotherapy.

The increased levels of CD68 and TLR4 in the peripheral nervous tissues of both BTZ- and VCR- treated animals are in line with the recent literature that suggests a fundamental role for neuropathy development of infiltrating macrophages in the peripheral nervous system (Montague and Malcangio, 2017). For example, it has been recently demonstrated that the intravenous immunoglobulin administration was able to reduce or prevent BTZ-induced heat and mechanical allodynia in rats by decreasing or preventing M1 macrophage infiltration in the peripheral nerves (Meregalli et al., 2018).

Interestingly, at the end of the chemotherapeutic schedule (BTZ day 28 and VCR day 14), we also detected significant changes in the central nervous system. In fact, a clear microglia activation was present in both BTZ- and VCR- treated mice, as shown by the upregulation of CD68 and CD11b.

Our results also support the literature which sustains the importance of astrocytes in CIPN (Stockstill et al., 2018). In fact, in our experiments, GFAP is the only marker that we found precociously activated in the spinal cord of BTZ-mice. This is in line with what recently suggested by Robinson and collegues, who demonstrated how the presence of mechanical hypersensitivity due to BTZ treatment correlated to an upregulation of GFAP (Robinson et al., 2014; 2015). Furthermore, at the end of the chemotherapeutic schedule, also VCR- mice showed a marked GFAP upregulation. At this time point the presence of cell stress/injury reached also the spinal cord as demonstrated by ATF3 upregulation in this nervous tissue.

The activation of the prokineticin system components was different in the two protocols. In fact in BTZ-mice no alteration of the PK system was evident at day 14, however at the end of the chemotherapeutic schedule (BTZ day 28) an upregulation of PK2 and its receptors was detectable in all tissues involved in pain transmission (sciatic nerve, DRG and spinal cord). On the other hand, VCR-mice showed an upregulation of PK-R1 and PK-R2 already at day 7 both in DRG and spinal cord, which was maintained until the end of the chemotherapy protocol (VCR day 14), when we also observed PK2 upregulation.

The differences in PK system activation in mice treated with BTZ and VCR could be related to several reasons such as the toxicity of the drug, the different mechanisms of action but also to the chemotherapeutic schedule used in our experiments.

From these results it is clear that the complete activation of the PK system in our model is delayed in comparison to painful symptoms appearance and the precocious neuroinflammation. This activation time of the PK system was unexpected for us, since in ours and other groups' previous works PK system activation well correlated with the onset of hypersensitivity (Castelli et al., 2016; Guida et al., 2015; Maftei et al., 2014). The results here presented demonstrated that in CIPN, prokineticins have an important role in sustaining, maintaining, and worsening hypersensitivity rather than in its development. However, PK system later activation could probably be linked to an amplification of DRG neuroinflammation and to the involvement of central nervous system.

This assumption is sustained by the fact that chronic treatment with PC1, even if it was started in the presence of an established hypersensitivity, was able to counteract the further decrease of mechanical and thermal thresholds, to protect and to reverse the established neuroinflammation, rebalancing pro- and anti- inflammatory cytokines both in the peripheral and central nervous system.

On the basis of the literature (Guida et al., 2015; Maftei et al., 2014) and of our immunofluorescence data that show how in peripheral nervous tissues of mice at the end BTZ schedule (day 28), CD68 + cells co-express PK2, we can suppose that infiltrating activated macrophages may represent an important source of PK2 in DRG and sciatic nerve even if we cannot exclude that other cell types, like satellite cells and neurons, may contribute to the *in vivo* PK2 increase.

We can hypothesize that during BTZ or VCR treatment, infiltrated and resident-activated immune cells, in association with satellite cells and Schwann cells, produce pro-inflammatory cytokines leading to a further recruitment of immune cells, like macrophages, into damaged nervous tissues.

These infiltrating macrophages not only produce PK2 but also express PK-Rs receptors (Martucci et al., 2008); hence, PK2 can act in autocrine or paracrine way sustaining a neuroinflammatory loop that worsen the neuronal damage and sustain a progressive glial activation at the spinal cord level (Guida et al., 2015).

The molecular mechanisms related to this process were not analysed in this study, but on the basis of the recent paper of Qu and colleagues (2019), a possible signaling pathway could be represented by STAT3 signaling. In fact, this transcription factor plays a crucial role in PK2 regulation and it was demonstrated how phosphorylated STAT3 can directly bind to the Pk2 promoter. Furthermore, a recent study (Liu et al., 2018) demonstrated that phosphorylated STAT3 levels were significantly increased after BTZ administration and that STAT3 activation in DRG contributes to CIPN. On the basis of these data, we can speculate that the activation of STAT3, consequent to pro-inflammatory cytokine increase in the peripheral nervous stations (Tsuda et al., 2011), could be one of the mechanisms behind PK2 upregulation.

The effect of the PK-Rs antagonist PC1 may be in part related to its ability to reduce macrophage activation and infiltration in the peripheral nervous stations and therefore to prevent pro-inflammatory signals and the consequent PK system upregulation which, based on our hypothesis, could then lead to an amplification of the neuroinflammatory condition. Obviously, this could not be considered the only mechanism of action of the PK-Rs antagonist. In fact, as also supported by the results on the acute anti-allodynic effect promoted by a single PC1 bolus, it can be hypothesized that not only PK2 acts on its receptors on immune cells but also on those expressed by neurons and glial cells enhancing pain pathway transmission (Negri et al., 2012) through TRPV1 and TRPA sensitization (Quartu et al., 2014; Vellani et al., 2006).

The effects on the peripheral nervous system induced by PC1 chronic administration have a strong impact also at the level of the spinal cord. In fact, by blocking the activation of the PK system with PC1, we prevent a further neuroinflammatory condition in the spinal cord. BTZ + PC1 and VCR+PC1-treated mice did not

show any increase of glial activation markers such as CD68, CD11b and TLR4 that were instead significantly enhanced in CIPN animals at the end of the chemotherapeutic schedule (BTZ day 28 and VCR day 14).
As already observed in other experimental models of neuropathic pain, IL-1β appear to be the main cytokine modulated in the spinal cord of BTZ-mice and the treatment with PC1 was able to prevent its upregulation.
We can hypothesize that in the spinal cord of mice undergoing chemotherapy, initially there is an early glial activation that is not related to PK system alterations. Once activated, glial cells start to produce PK2, as demonstrated by the colocalization of PK2 and GFAP signals in the immunofluorescence experiments, thus confirming what we already observed in another experimental model of neuropathic pain (Maftei et al., 2014). PK2 promotes a further glial activation and cytokine alterations that may participate in central sensitization processes. The antagonism of the PK system probably prevents, also in the central nervous system, a further activation/signal amplification. However, while microglia was modulated by PK-Rs antagonism, astrocyte activation was not reverted by PC1 chronic treatment and may be responsible for the only partial anti- hyperalgesic effect observed in PC1 treated mice.
All together, our data on the peripheral and central nervous system, clear support the well-known flow of neuroimmune activation that goes from the peripheral to the central nervous system (Calvo et al., 2012; Sacerdote et al., 2013; Scholz and Woolf 2007), which is at the basis of neuropathic pain development and maintenance and they underline the important role of the prokineticin system in this sensitization process.
Finally, the data reported in this study could have translational implications. First of all, considering that CIPN develops in about 1/3 of patients undergoing chemotherapy, PC1 may be administered only when the symptoms have appeared allowing patients to continue the chemotherapeutic treatment. In addition, we show that PC1 has a protective role in a two-cycle BTZ/ VCR schedule: in fact, in the second cycle, the allodynic effect induced by the chemotherapeutic drugs is less evident in mice previously treated with PC1 if compared to that observed in BTZ/VCR-only re-treated mice. Furthermore, the second PC1 treatment completely normalizes the mechanical thresholds. Considering that patients often undergo multiple chemotherapeutic cycles, the protective role exerted by PC1 on a second chemotherapeutic cycle could be clinically relevant in order to slow down the re-appearance of the side effect. We plan to deeply investigate the reason behind this protective effect of PC1 in future studies. At the moment, we can only speculate that PK-Rs antagonist effect could be due to its ability to counteract neuroinflammation and thus blocking a further tissue damage. It can be hypothesized that chronic PC1 treatment may induce long-lasting modification in the PK system or enhance protective mechanisms that may be important in a second chemotherapeutic cycle, but we are aware of the fact that further experiments are needed to sustain this possibility.

In the second part of our project we decided to focus our attention on the neuronal component related to CIPN and the possible effects of PK antagonism. It is important to notice that PK system interaction with the immune system in neuropathy has been studied in different models of neuropathic pain, while at the moment there are few published studies about PK system and neurons in this pathological condition. For this reason and given the involvement of neuronal cells in CIPN, we decided to start this line of research by using an *in vitro* model represented by DRG primary sensory neurons.
It is now well accepted that DRG sensory neurons and their axons, but also satellite cells, are very susceptible to the damage induced by antineoplastics because of the absence of the blood-brain barrier and the presence of fenestrated capillaries which lead to an unconstrained drug permeation (Lees et al., 2017). For this reason, in addition to *in vivo* animal models, numerous *in vitro* studies have been carried out, mainly on dorsal root ganglia cell cultures, in order to investigate the molecular mechanisms of chemotherapy neurotoxicity.

Since our previously described results confirmed the widely known susceptibility of DRG to toxic agents, we investigated bortezomib and vincristine effects on DRG primary sensory neurons. In particular, we focused our attention on neurite outgrowth, an important morphological phenotype of neuronal cells that correlates with their function and health (da Silva and Dotti, 2002).

It is important to note that dissociated DRG cultures are not purely neuronal but also satellite cells and a small percentage of macrophages are present. Pure neuronal cultures can only be obtained by using anti-mitogen agents, but this is considered a toxic treatment that can interfere with neuronal viability. Furthermore, the presence of satellite cells in DRG cultures more closely resembles the *in vivo* environment of sensory neurons (Alè et al., 2015).

In the present study, we used DRG collected from healthy adult C57BL/6J wildtype mice, 9 weeks old, to be closer to our *in vivo* model and also to the clinical situation (Staff et al., 2013).

We began to analyze the effect of the proteasome inhibitor bortezomib on cultured primary sensory neurons. In our protocol DRG cell cultures were exposed for three days to different concentration of bortezomib (Alè et al., 2015). Neurons exposed to bortezomib showed a concentration-dependent reduction in neurite outgrowth. In order to test the possible *in vitro* effect of the PC1, a range of concentrations of the PK-Rs antagonist were co-applied (Severini et al., 2015) to Bortezomib 6nM, a concentration in which a reduction of the 50% of neurite length was detected. PC1 concentration-dependently reversed BTZ-toxicity. Given the similarities between BTZ and VCR mechanisms involved in CIPN observed during the *in vivo* experiments, the *in vitro* effect of PC1 was tested also on VCR treated primary sensory neurons. Since the literature was really heterogeneous regarding the *in vitro* doses of vincristine and the time of incubation (Guo et al., 2017; Miyano et al., 2009), a wide range of VCR concentrations was tested, using the same timing of exposure of the BTZ protocol.

Also on cultured neurons, as it happened in the *in vivo* experiments, the two chemotherapeutic drugs despite the different antineoplastic mechanism of action, induce a similar effect on neurons. In fact, we detected a dose dependent reduction of cellular processes also in this case. However, if comparing the effect of similar doses of the two drugs, VCR seemed to be more neurotoxic than BTZ. This confirms the clinical data where VCR, due to its high toxicity, induces CIPN in a larger number of patients compared to BTZ.

Four concentrations of VCR able to induce a significant alteration in neurites arborization were tested in association to PC1 250nm. PK-Rs antagonist was effective in all of them except in the 50nM probably due to the high dose.

On the basis of our data the preventive effect of PC1 could be due from one side to the prevention of neurite loss, since at 24h some neurites are already formed, and on the other hand by contrasting the chemotherapy toxicity it can allow new processes formation.

In order to start to address the molecular mechanisms at the basis of this positive effect of PC1, we choose to analyse biochemical parameters of cells treated with 1nM VCR because it was the highest dose of VCR where PC1 was still effective. PK system alterations and inflammatory parameters were analysed in cells treated with VCR 1nM with or without PC1 250nM.

The molecular mechanisms at the basis of this effect have still to be studied but on the basis of our *in vivo* and *in vitro* experiments we can hypothesize that VCR induces a neurotoxic damage, as demonstrated by the increased levels of ATF3. The chemotherapeutic agent is able to directly bind to the TLR4 (Montague and Malcangio, 2017; Ustinova et al., 2013), which triggers the activation of several pro-inflammatory pathways. The upregulation of pro-inflammatory cytokines, such as IL-6 and IL-1β, can on the other hand induce the activation of STAT3 that binds to the pk2 promoter and lead to the activation and further production of PK2 (Qu et al., 2018), which is demonstrated by the increased levels of PK2 in VCR-cells. Once activated, PK2 is able to sustain its own production in a positive loop and to amplify the pro-inflammatory

signal as demonstrated by several *in vivo* and *in vitro* studies (Negri and Maftei, 2017; Giannini et al., 2009; Monnier and Samson 2008; Martucci et al., 2006). This pathway, as previously mentioned, it is probably amplified in the *in vivo* models where infiltrating immune cells can participate to this process.
Our preliminary immunohistochemical analysis, showed the presence of PK2 not only on neurons but also on other cells present in the DRG cultures, which probably are satellite cells. This is in accordance with other studies that show the presence of PK2 and its receptors both on neurons and surrounding accessory cells (Severini et al., 2015; Caioli et al., 2016). Furthermore, also in our *in vivo* analysis, in DRG we detected the presence of PK2 signal not uniquely in association to CD68, but also in other areas, leading to the hypothesis that PK2 is expressed also by other cell types, such as neurons and satellite cells. At the moment, we do not know if the upregulation of PK2 mRNA is concurrent to an increase in its protein levels and the same applies for the cell types responsible for PK2 expression increase. However, we will deeply investigate this aspect in our future works. However, on the basis of PK system distribution, it can be inferred that several diffent mechanisms could be implicated in the action of Prokineticin, as for example TRPV and TRPA signaling, in fact these channels have been shown to be functional associated with PK2 in DRG neurons (Vellani et al., 2016).
When the effect of PC1 250nM alone was tested in the cell culture, no morphological or biochemical alteration was detected. This is in accordance with our *in vivo* data where the administration of PC1 alone was not able to exert any effect on pain-like behavioural tests and cytokine balance (Castelli et al., 2016). Interestingly, in these cell cultures PC1 exerted a preventive effect because it was administered together with chemotherapy and not once the damage was already there.
The presence of PC1 was able to antagonize not only the upregulation of the PK system components but also the alterations in the expression of cytokines and of TLR4. As well as the mechanisms involved in the activation of PK system due to chemotherapy, also PC1 effect could be linked to several signaling pathways and further analysis are needed. For the moment, on the basis of our results we can suppose that PK-Rs antagonist, by binding to its receptors is able to reduce or even stop the amplification of damage.
The preventive effect exerted *in vitro* by PC1 is in accordance with what observed in the *in vivo* diabetic neuropathy model where PC1 was effective not only in a therapeutic way but also in a preventive one (Castelli et al., 2016). These results give us an idea of the possibility of testing PC1 also in a preventive way in our *in vivo* model, supporting the data that we obtained in the prevention of hypersensitivity exerted by PC1 on the second chemotherapeutic cycle. This could be particularly interesting from a clinical point of view for those patients who are at higher risk of developing CIPN because of pre-existing conditions (diabetes and obesity), lifestyle choices (smoking and chronic use of alcohol) and genetic predisposition (Boyette-Davis et al., 2018).

In conclusion, PK system seems to be involved in the mechanisms at the basis of chemotherapy-induced peripheral neuropathy. Blocking PK2 signalling by using PC1 appears as a promising therapeutic approach as this molecule not only reduces painful symptomatology but also, acting on several cell types, prevents and suppresses the causes underlying disease progression as neuroinflammation. Therefore, we believe that PK system could be considered a valid target for the development of novel pharmacological approaches to manage CIPN and, more in general, neuropathic pain conditions.

8. References

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9. Publications and other activities

Nov 2017 – Jun 2018: Visiting PhD Fellow
Dept. of Physiology and Medical Physics, Medical University of Innsbruck – Austria
Advisor: Prof. Michaela Kress
Analysing the effects of different chemotherapeutic agents and Prokineticin system antagonist on neurite outgrowth of dorsal root ganglia primary sensory neurons.

Publications

- Moschetti G., Amodeo G., Paladini M.S., Molteni R., Balboni G., Panerai A.E., Sacerdote P., Franchi S.; (2019) *Prokineticin 2 promotes and sustains neuroinflammation in vincristine treated mice. Focus on pain and emotional like behavior*. Brain Behavior Immun S0889-1591 (19) 30492-1.

- Moschetti G., Amodeo G., Maftei D., Lattanzi R., Procacci P., Sartori P., Balboni G., Onnis V., Conte V., Panerai A., Sacerdote P., Franchi S.; (2019) *Targeting prokineticin system counteracts hypersensitivity, neuroinflammation and tissue damage in a mouse model of bortezomib induced peripheral neuropathy.* J Neuroinflammation 16(1):89.

- Mitrić M., Seewald A., Moschetti G., Sacerdote P., Ferraguti F., Kummer K., Kress M.; (2019) *Layer- and subregion-specific electrophysiological and morphological changes of the medial prefrontal cortex in a mouse model of neuropathic pain.* Sci Rep. 9(1):9479.

- Amodeo G., Bugada D., Franchi S., Moschetti G., Grimaldi S., Panerai A., Allegri M., Sacerdote P.; (2018) *Immune function after major surgical interventions: the effect of post-operative pain treatment.* J Pain Res. 11:1297-1305.

- Vellani V., Moschetti G., Franchi S., Giacomoni C., Sacerdote P., Amodeo G.; (2017) *Effects of NSAIDs on the release of Calcitonin Gene-Related Peptide and Prostaglandin E2 from rat trigeminal ganglia.* Mediators Inflamm. 2017: 9547056.

- Brini A.T., Amodeo G., Ferreira L.M., Milani A., Niada S., Moschetti G., Franchi S., Borsani E., Rodella LF, Panerai A.E., Sacerdote P.; (2017) *Therapeutic effect of human adipose-derived stem cells and their secretome in experimental diabetic pain.* Sci Rep. 7(1):9904.

- Abbate G.M., Mangano A., Sacerdote P., Amodeo G., Moschetti G., Levrini L.; (2017) *Substance P expression in the gingival tissue after upper third molar extraction: effect of ketoprofen, a preliminary study.* Journal of biological regulators & homeostatic agents 31(1):239-244.

- Franchi S., Amodeo G., Gandolla M., Moschetti G., Panerai A.E., Sacerdote P.; (2017) *Effect of Tapentadol on splenic cytokine production in mice.* Anesth Analg. 124(3):986-995.

Chapters in books:

- Sacerdote P.G., Moretti S., Franchi S., **Moschetti G.**; (2018) *Long term effects of Delta-9 tetrahydrocannabinol exposure in adolescence on peripheral central cytokines.* Advances in Psychobiology, Chiappelli F., Nova Science Publisher.

Conferences

- **Moschetti G.,** Amodeo G., Kalpachidou T., Paladini M.S., Molteni R., Kress M., Sacerdote P., Franchi S.; "Involvement of Prokineticin system in a mouse model of vincristine-induced peripheral neuropathy". XVIII Congresso Nazionale SINS (Società Italiana di Neuroscienze), Perugia 26-29th September 2019. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Kalpachidou T., Kress M., Sacerdote P., Franchi S.; "Prokineticin system as a new target to counteract experimental Vincristine induced peripheral neuropathy". 7th International Congress on Neuropathic Pain (NeuPSIG 2019), London – May 9-11th, 2019. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Niada S., Savadori P., Franchi S., Brini AT., Sacerdote P.; "Intraplantarly/intravenously injected secretome from human adipose mesenchymal stromal cells reduce thermal and mechanical hyperalgesia in the monosodium-iodoacetate osteoarthritis model in mice". GISM 2019 Annual Meeting Congress, Genova 4-5th April 2019. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Balboni G., Sacerdote P., Franchi S.; "Role of Prokineticin system in Vincristine-induced peripheral neuropathy in mouse: focus on gender". More than neurons, Turin - November 29 – December 1st, 2018. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Maftei D., Kalpachidou T., Lattanzi R., Kress M., Sacerdote P., Franchi S.; "Prokineticin system's antagonism: implications in a mouse model of chemotherapy induced peripheral neuropathy". XXI SIF SEMINAR - PhD Students, Fellows, Post Doc and Specialist Trainees, Bresso (MI) – September 19-22th, 2018. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Maftei D., Lattanzi R., Sacerdote P., Franchi S.; "Prokineticin system: a new target to counteract experimental chemotherapy-induced peripheral neuropathy". 11th FENS Forum of Neuroscience, Berlin – July 7-11th, 2018. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Maftei D., Kalpachidou T., Lattanzi R., Kress M., Sacerdote P., Franchi S.; "Understanding Prokineticin system's role in a mouse model of chemotherapy induced peripheral neuropathy". 9th Next Step Edition, Milan – July 4th, 2018. *Oral Presentation.*

- **Moschetti G.**, Amodeo G., Maftei D., Lattanzi R., Sacerdote P., Franchi S.; "Investigating the role of prokineticin system in a murine model of chemotherapy-induced peripheral neuropathy". European Pain School (EPS 2018), Siena – June 10-17th, 2018. *Oral Presentation.*

- **Moschetti G.,** Amodeo G., Lattanzi R., Sacerdote P., Franchi S.; "Role of Prokineticin system in an experimental model of chemotherapy-induced peripheral neuropathy". 38°Congresso Nazionale della Società Italiana di Farmacologia, Rimini – October 24-28, 2017. *Poster presentation.*

- **Moschetti G.**, Amodeo G., Panerai A.E., Sacerdote P., Franchi S.; "Targeting Prokineticin System to counteract experimental chemotherapy-induced peripheral neuropathy". 8th Next Step Edition, Milan – June 29, 2017. *Oral Presentation.*

- **Moschetti G.**, Amodeo G., Lattanzi R., Sacerdote P., Franchi S.; "Antagonism of the Prokineticin system counteracts chemotherapy induced peripheral neuropathy in mice". Monothematic SIF congress on "The Pharmacological Basis of Novel Pain Therapeutics"– Firenze, May 4-5, 2017. *Oral Presentation.*

Awards

- Best Poster Award: SINS 2019 XVIII National Congress, Perugia 26-29th September 2019.
- Best Poster Award: GISM 2019 Annual Meeting Congress, Genova 4-5th April 2019.
- Hellobio Travel Grant for the 7th International Congress on Neuropathic Pain (NeuPSIG2019), London – May 9-11, 2019.
- Bio-techne Travel Grant for the 11th FENS Forum of Neuroscience, Berlin – July 7-11, 2018.
- Selected for the participation to the European Pain School (EPS 2018), "Pain from fetus to old age", Siena – June 10-17, 2018.
- Best Poster Award: 38°Congresso Nazionale della Società Italiana di Farmacologia, Rimini – October 24-28, 2017.
- YAP (Young Against Pain) Selected project: IX SIMPAR- ISURA Congress (Study in Multidisciplinary Pain Research) – Firenze – March 29- April 1st 2017. Moschetti G.; *"Investigating the role of Prokineticin System in the pathobiology of chemotherapy-induced peripheral neuropathy".*

Advanced courses:

- Corso introduttivo alla sperimentazione animale; Università degli Studi di Milano; September 16-18, 2019.
- Sperimentazione animale – Corso Base: Dal concetto delle 3S alla normativa vigente (FAD) - IZSLER Brescia; corso online – Agosto 2019.
- Abuso degli oppioidi per il dolore cronico– Italian Society of Toxicology (SITOX); Milan, January 21, 2019.
- IX SIMPAR- ISURA Congress (Study in Multidisciplinary Pain Research) ; Firenze – March 29- April 1st 2017.
- Corso di Formazione AiFOS, Corso di Formazione/Aggiornamento professionale, Prevenzione dei rischi Chimico e Biologico in Laboratorio; University of Milan - January 19, 2017.
- Corso Introduttivo alla Sperimentazione Animale; I.RC.C.S. Mario Negri, Milan, November 16-18, 2016.
- GISM (Gruppo Italiano Staminali Mesenchimali) Annual Meeting; Brescia– October 20-21, 2016.
- CEND Monothematic Meeting - Neuroinflammation in CNS health and disease (CEND: Center of Excellence on Neurodegenerative Diseases); Università degli Studi di Milano; October 19, 2016.
- on Neurodegenerative Diseases); Università degli Studi di Milano; October 19, 2016.
- Cochrane e altre risorse EBM ; Università degli Studi di Milano, October 18, 2016.

Other activities:

Experimental thesis co-advisor :

- "*Role of Prokineticin system in the development of peripheral neuropathy induced by Vincristine: focus on the DRG*"; Candidate: F. Colombi, Master Degree in Biology Applied to Research in Biomedicine – Università degli Studi di Milano, December 2018. Advisor: Prof.ssa Paola Sacerdote.
- "*Role of Prokineticin system in chemotherapy-induced peripheral neuropathy*"; Candidate: E. Henke, Master Degree in Biology Applied to Research in Biomedicine – Università degli Studi di Milan, April 2017. Advisor: Prof.ssa Paola Sacerdote.