Prokineticin 2 promotes and sustains neuroinflammation in vincristine treated mice: focus on pain and emotional like behavior

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Abstract

Vincristine (VCR) treatment is often associated to painful neuropathy. Its development is independent from antitumoral mechanism and involves neuroinflammation. We investigated the role of the chemokine prokineticin (PK)2 in a mouse model of VCR induced neuropathy using a PK-receptors (PK-R) antagonist to counteract its development. We also evaluated emotional like deficits in VCR mice.

VCR (0,1 mg/kg) was i.p. injected in C57BL/6J male mice once a day for 14 consecutive days. Pain, anxiety and depressive like behaviors were assessed in animals. PK2, PK-Rs, cytokines, neuroinflammatory markers (CD68, CD11b, GFAP, TLR4) and ATF3 were evaluated in DRG, spinal cord, prefrontal cortex and hippocampus. The PK-Rs antagonist PC1, was s.c. injected (150µg/kg) twice a day from day 7 (hypersensitivity state) until day 14. Its effect on pain and neuroinflammation was evaluated.

VCR mice developed neuropathic pain but not mood alterations. After 7 days of VCR treatment we observed a neuroinflammatory condition in DRG with high levels of PK-Rs, TLR4, CD68, ATF3 and IL-1β without relevant alterations in spinal cord. At day 14, an upregulation of PK system and a marked neuroinflammation was evident also in spinal cord. Moreover, at the same time, we observed initial alterations in supraspinal brain areas. PC1 treatment significantly counteracted neuropathic pain and blunted neuroinflammation.

Highlights

Vincristine treated mice develop neuropathic pain but not mood alterations.
Prokineticins are involved in the progression and worsening of hypersensitivity.
Prokineticin antagonism counteracts pain and neuroinflammation.

PK system is a promising pharmacological target to contrast chemotherapy-induced neuropathy.

Keywords

prokineticins, neuropathic pain, neuroinflammation, vincristine, mood alterations

1. Introduction

Vincristine (VCR) is one of the most common antineoplastic drugs for the treatment of several cancer types i.e. leukemia, lymphoma and sarcoma (Gidding et al., 1999). However, its clinical use is related to several side effects, including peripheral neuropathy, thus leading to treatment discontinuation. VCR antineoplastic effect is based on the disaggregation of tubulin monomers during mitosis that inhibits cell division (Lobert et al., 1996; Martino et al., 2018). It is now accepted that the development of peripheral neuropathy is not strictly related to drug primary antitumoral effect, but converges on common several downstream pathways involving different players (Carozzi et al., 2015; Boyette-Davis et al., 2015). A crucial role of non-neuronal cells, like macrophages and glial cells, is now recognized and several studies suggest an association between increased macrophage infiltration in dorsal root ganglia (DRG) and peripheral nerves and pain like behavior appearance in experimental animals (Peters et al., 2007; Kiguchi et al., 2009; Montague and Malcangio, 2017). In particular in CIPN activated macrophages and glial cells, like microglia and astrocytes, have been shown to release proinflammatory cytokines and chemokines such as IL-1β, TNFα, IL-6, CCL2 and CXC family members (Ledeboer et al., 2007; Kiguchi et al., 2008; Muthuraman et al., 2011; Montague et al., 2018), leading to neural excitability and pain transmission enhancement. Furthermore, the same cytokines, when increased in the supraspinal areas, i.e. prefrontal cortex and hippocampus, can start and sustain a neuroinflammatory condition related to the presence of altered affective-like or cognitive behavior (Haroon and Miller 2017; Dantzer 2018; Pfau et al., 2018; Bhattacharya and Jones, 2018; Santos and Pyter, 2018;). Both pain and antitumoral drugs can play a role in the development of altered affective behavior; in fact patients with chronic pain or

receiving chemotherapy often experience changes in mood, including the presence of anxiety and depression, that further impact their life quality. (Wefel and Schagen, 2012; Santos and Pyter, 2018). In accordance with the above described crucial role of the neuroinflammatory component in CIPN, we recently suggested (Moschetti et al., 2019) a role of the newly discovered chemokine family, the prokineticin (PK) family in the peripheral neuropathy induced by the proteasome inhibitor bortezomib (BTZ). PK family is composed by two proteins: PK1 and PK2 and by two G protein coupled receptors, PK-R1 and PK-R2. PK-Rs are widely distributed in pain stations like DRG and spinal cord. In DRG PK-R1 and PK-R2 are expressed in different types of neurons: in particular, 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 suggesting that these receptors might be involved in central transmission of the nociceptive signal (Negri and Lattanzi, 2012). In non neuronal cells, such as astrocytes and microglia PK-R1 is mostly expressed. Moreover, this receptor is more abundant than PK-R2 also on human and rodent monocytes/macrophages.

It is known that PK-Rs may be coupled to Gq, Gi, and Gs (Lin et al., 2002; Soga et al., 2002) depending on their cellular localization and can activate different intracellular signal pathways (for an explicative review, see Negri and Ferrara, 2018). In the monocytes/macrophage/microglia lineage, in DRG and spinal cord they are Gq-coupled receptors and promote intracellular Ca^{2+} mobilization through the activation of phospholipase C (PLC) and formation of IP3. In the DRG, PKR activation, via Gq, increases intracellular calcium and induces protein kinase C (PKC)-ε translocation to the plasma membrane (Vellani et al., 2006). Regarding the ligands, PK2 is recognized as an important player at cross road between

inflammation and pain (Franchi et al., 2017) while PK1, that is not expressed in DRG and spinal cord, is more involved in angiogenesis. It is known that PK2 can modulate the immune function inducing a proinflammatory phenotype and sustaining a proinflammatory condition (Martucci et al., 2006). Moreover, it sensitizes TRPV1 and TRPA1-expressing nociceptors (Negri et al., 2006; Vellani et al., 2006) and induces the release of proinflammatory/ pro-algogen mediators like cytokines, Substance P (SP) and CGRP. Furthermore, PK2 is involved in the development of experimental neuropathic pain (Maftei et al., 2014; Guida et al., 2015; Castelli et al., 2016) of different origin, including BTZ induced neuropathy.

Interestingly, a role for PK2 in mood regulation has also been described. Intracerebroventricular injection of PK2 leads to increase of anxiety and depressive like behavior while mice deficient in PK2 display reduced anxiety and depression (Li et al., 2009).

Considering the above described involvement of PK2 in the development of experimental pain and anxiety/depressive like behavior, the present study aims to:

- assess the role of PK system in vincristine (VCR) induced peripheral neuropathy and verify if the blockade of PK-Rs can counteract VCR induced hypersensitivity, representing a generalized strategy to contrast chemotherapy-induced neuropathy,
- verify if, in our experimental model of VCR induced neuropatic pain, the presence of hypersensitivity is related to the presence of altered affective behaviors.

2. Materials and Methods

An explicative timeline of the experimenthal design is shown in Figure 1.

2.1 Animals

Male C57BL/6J mice (Charles River Laboratories, Calco, Italy), nine-weeks-old, were individually housed with 12 hours light/dark cycles, temperature of 22 ± 2 °C, humidity of 55 ± 10%, food and water *ad libitum*. Animals were acclimatized to the environment for ten days and subsequently handled (exposure to a passive hand, tickling, hand restraint for few minutes) for 3 consecutive days before starting the experiments. All animal experiments comply with ARRIVE guidelines and were carried out in accordance with EU directive 2010/63/EU for animal experiments and were approved by the Animal Care and Use Committee of the Italian Ministry of Health (permission number 709/ 2016 to SF).

2.2 Drugs

Mice were randomly divided in two experimental groups, controls (CTR) and vincristine (VCR) treated mice by using the physical method of coin flipping.

Vincristine sulfate (TOCRIS Bioscience™, Minneapolis, USA), diluted in saline solution (0,9% NaCl) was intraperitoneally (i.p) injected at the dose of 0,1 mg/kg/ day for 14 consecutive days. The vincristine dose was assumed from the literature (Kiguchi et al., 2008). The schedule treatment allows to induce a detectable painful neuropathy minimizing unspecific systemic toxicity and preventing mice death. Control mice were treated with saline solution.

VCR neuropathic mice were randomly selected (coin flipping method) for PK-Rs antagonist (PC1) treatment. PC1, a triazine guanidine derivative selective for PK-Rs

(Balboni et al., 2008), was firstly dissolved in DMSO 10% and then subcutaneously administered at the dose of 150 µg/kg, diluted in saline solution, two times/day for 7 consecutive days: from day 7 until the end of the VCR schedule (day 14). The chosen dose represents the most effective one in contrasting pain on the basis of the results of a dose-finding study performed in CCI neuropathic mice (Maftei et al., 2014). The same dose was also effective in other neuropathic pain models like nerve injury model (SNI), streptozotocin induced diabetes and bortezomib induced peripheral neuropathy (Guida et al., 2015; Castelli et al., 2016; Moschetti et al., 2019). In order to minimize possible interactions between the two drugs, mice were always treated with VCR early in the morning (around 8:00 a.m.) and they were then treated with PK-Rs antagonist around 12:00 p.m. for the first injection and at 6:00 p.m. for the second PC1 administration. The effect of PC1 on nociception and related biochemical parameters (cytokines, PK2 and PK-Rs in nervous tissues) in naïve mice has been previously charaterized (Castelli et al., 2016; Maftei et al., 2014; Giannini et al., 2009) showing that the antagonist alone did not affect these aspects in normal mice. Therefore according to 3R guidelines we did not include this experimental group in the present work.

2.3 Behavioral evaluations

2.3.1 Pain-related behavioural evaluations

All behavioral evaluations were performed by researchers blind to treatments. Behavioral tests were performed after an habituation period of 30 minutes. Responses to mechanical and thermal stimuli were assessed before starting VCR protocol (0), and after 3, 7, 11 and 14 days of chronic VCR treatment. Considering the time span of the acute effect of PC1 (Castelli et al., 2016; Moschetti et al., 2019),

to test the effect of the chronic treatment with PC1, all behavioral tests (day 11 and 14) were performed at least 12 hours after the last injection of the antagonist.

For hypersensitivity tests for each mouse we performed three different measurements on each hind-paw, the values were then averaged obtaining a single value. The values obtained from each mouse of the same experimental group were then averaged and used for statistical analysis.

2.3.1.1 Von Frey Test

Mechanical allodynia 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). Animals were placed in a plexiglass test cage (w 8.5 x h 8.5 cm) upon a metallic mesh and the rigid tip of a Von Frey filament (punctate stimulus) was applied to the skin of the mid-plantar area of the hind paw with increasing force (ranging up to 10 g in 10 s), starting below the threshold of detection and increasing until the animal feels pain and it removes its paw. The withdrawal threshold (PWT) was expressed in grams.

2.3.1.2 Acetone Drop Test

Cold allodynia was evaluated as previously described (Flatters and Bennet, 2004; Brini et al., 2017; Moschetti et al., 2019). A drop (50 µl) of acetone was placed in the middle of the plantar surface of the hind paw and mouse behavior was initially monitored, inside a plexiglass arena (h50 x w 40), for 20 s. If mice did not withdraw, flick or stamp the hind paw within this time lapse, no other observations were made and the researcher assigned the score 0 to mice. However, if within this period the animal responded to the cooling effect of acetone, its behavior was assessed for an

additional 20 s. Responses to acetone were graded (allodynia score), using a 4 points scale, as follow: 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking (more than twice) of the paw; 3, repeated flicking of the paw with licking directed at the plantar surface of the hind paw.

2.3.1.3. Plantar Test

Thermal hyperalgesia was tested according to the Hargreaves procedure (Hargreaves et al, 1988) slightly modified by us for mouse (Franchi et al., 2012), using a Plantar test apparatus (Ugo Basile, Comerio, Italy). Briefly, mice were placed in a plexiglass cubicles (w 11 x h 11 cm) and a constant intensity radiant heat source (beam diameter 0.5 cm and intensity 20 I.R.) was aimed at the mid plantar area of the hind paw until the animal removed its paw. Paw withdrawal latency (PWL) that is the time, in seconds (s), from initial heat source activation until paw withdrawal was recorded.

2.3.2 Mood-related behavioural evaluations

Mice were randomly divided (coin flipping) in two cohorts to assess the presence of anxiety or depressive like behavior associated to VCR treatment. In this way half of the animals underwent tests to assess the development of depression-like phenotypes (Depression-like Cohort) and the other half tests to measure anxiety-like behaviors (Anxiety-like Cohort).Anxiety and depression were assessed at the end of VCR schedule (day 14). Mice were habituated in the testing room for 1 hour. Evaluations were performed/ analysed by researchers blind to treatments.

<u>Anxiety-like behavior</u>

2.3.2.1 Open Field Test (OFT)

Open field test was performed to score the mice locomotor activity. Mice were placed in an unfamiliar open arena (40x40x50 cm) and allowed to freely explore for 10 min. After this session they were returned to their cages. The same protocol was used for the baseline measurement and the test session (day 14). The session was recorded and then the videos were analyzed by three experimenters blind to the treatment. The mobility of the mice was scored during the entire session.

2.3.2.2 Marble Burying Test

Cages were filled with approximately 5cm deep with wood chip bedding, lightly tamped down to make a flat surface. A regular pattern of 18 glass marbles was placed on the surface, evenly spaced, each about 3cm apart. Mouse was placed in each cage and left in it for 30 minutes. After this time span, mouse was put back in its cage and the number of marbles buried with bedding (2/3 their depth) was counted (Deacon, 2006).

2.3.2.3 Novelty Suppressed Feeding (NSF)

The test was performed by scoring the latency to feed for a food-deprived mouse when it is introduced to an unfamiliar and adversive environment. Briefly, prior to the test, individually housed mice were subjected to a 24h food deprivation. Mice were weighed before and after the food deprivation to check differences in the loss of the body weight between experimental groups. Novelty-suppressed feeding assay was performed in a open arena with bedding covering the floor and a food pellet in the center, placed on a Petri dish under bright light. Each mouse was removed from its home cage, weighed, and introduced to one corner of the arena. The mouse was allowed to freely investigate the arena and the food for a maximum of 5 min. Feeding behavior was recorded by a video camera and the latency to biting the food was

scored later by two experimenters blind to the treatment. Once the mouse bit the food, it was quickly transferred to the home cage with a new pre-weighed pellet of food. The mouse was undisturbed in the home cage for 5 min and then the food was removed and weighed again.

2.3.2.4 Dark/Light Box (DLB)

The test was conducted in a two-chamber arena (40x40x50 total cm) with a black divider. The light chamber measures 28x40x50 cm while the dark chamber 12x40x50. An opening was located at the center bottom of the divider. The walls of dark chamber were made of black plexiglass while those of the other chamber were white. The lid of the dark box was made of a black plexiglass. During the test mice were first placed into the dark chamber and allowed to freely travel between the chambers for 5 min. The entire session was video recorded and later analyzed by three experimenters blind to the treatment. The time spent in the light chamber, and the number of transitions (defined as the times the animal passes in or out of the light and dark chambers) were scored.

Depressive-like behavior

2.3.2.5 Forced Swim Test (FST)

The assay was performed using a standard protocol of forced-swim test for mice to test the “behavioral despair” at the end of the treatment. Briefly, a 3L glass beaker (14.5 cm diameter, 27 cm height) filled with water (23-25 °C) was used as apparatus. Mice were placed in the beaker and allowed to swim undisturbed for 6 min. The animals were then removed, dried, and returned to their home cages. Water was changed between each subject. The entire session was recorded with a video

camera. Later, the videos were scored by three experimenters blind to the treatment for the duration of immobility during the last 4 min of the session.

2.3.2.6 Sucrose Preference Test (SPT)

Sucrose preference test was used as in indicator of anhedonia. During habituation, mice were given 48 hours of continuous exposure to two regular bottles, both containing tap water (regular water), in their home cages with ad libitum access to food. Mice were then exposed to two bottles (sucrose/water during baseline measurements and sucrose preference test and water/water for the remaining days) in their home cage during all the experiment. After the habituation, sucrose consumption baseline was measured one week before vincristine treatment, in two consecutive sessions during the dark phase. In the baseline measurement stages, each mouse was given one bottle of 2% sucrose solution and one of regular water for 16 hours (6 P.M. to 10 A.M.). Mice were tested at the end of vincristine treatment with two consecutive 16 hours sessions during the dark phase. During the test, all animals were exposed to one bottle of 2% sucrose solution and one of regular water, both weighed before and after the test and switched in the position to reduce any confound produced by a side bias. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake for each tested animal.

2.4 Tissue collection

After 7 days of chronic VCR treatment and the end of VCR/PC1 schedule (day 14, VCR) mice were euthanized by CO_2 inhalation. DRG (L4-L6), lumbar (L4-L6) spinal cord, prefrontal cortex (PFC) and hippocampus (HPC) were dissected, frozen in liquid nitrogen and stored at -80°C until further processing.

2.5 RNA extraction and RT-qPCR

Total RNA was isolated from DRG (L4-L6), lumbar spinal cords, PFC and HPC using TRIzol® reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instructions and re-suspended in a volume of 10–25 µl, depending on the tissue, of RNase-free water. RNA quantity and quality was determined using a BioPhotometer (Eppendorf, Germany). Reverse transcription of mRNA was performed according to the manufacturer's instructions (iScript cDNA Synthesis Kit, Bio-Rad).

Gene of interest were analyzed by Reverse transcription quantitative polymerase chain reaction (RT-qPCR) using the following TaqMan Gene Expression Assays (Thermofisher Scientific, Waltham, USA): Prokineticin2 (Mm01182450_g1), Prokineticin receptors (Prokr1 Mm00517546_m1; Prokr2 Mm00769571_m1), cytokines (TNF-α Mm00443258_m1; IL-6 Mm00446190_m1; IL-1β Mm00434228_m1; IL-10 Mm00439616_m1;), CD68 (Mm_03047343), CD11b (Mm00434455_m1), TLR4 (Mm00445274_m1), GFAP (Mm01253033_m1) and ATF3 (Mm00476033_m1) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH Mm99999915_g1). 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 30seconds 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.

2.6 Statistical Analysis

Data are expressed as mean± SEM. Results obtained from pain related behavioral evaluations were analyzed using Two way-ANOVA analysis of variance with repeated measures (factors: treatments and time) followed by Bonferroni's test for multiple comparisons.

Anxiety and depressive like behaviours results and biochemical evaluations performed at day 7 were analyzed by using unpaired two tails *t*-test while biochemical data performed at day 14 were tested using One way-ANOVA followed by Bonferroni's test. Statistical analysis was performed using GraphPad 6 (San Diego, CA). Differences were considered significant at $p \leq 0.05$.

3. Results

3.1 Effect of VCR treatment on hypersensitivity (allodynia and hyperalgesia) and affective behavior (anxiety and depressive like behavior). Effect of PK-Rs antagonist (PC1) on hypersensitivity.

VCR induced in mice a progressive reduction of both mechanical and thermal thresholds. As shown in figure 2 (panels a, b and c) VCR treatment induced in mice the presence of an allodynic and hyperalgesic state characterized by a significant reduction of mechanical and thermal thresholds evident from day 3 until the end of VCR administration (panel a, b and c; CTR vs VCR $p< 0.0001$).The treatment with the PK-Rs antagonist PC1 was started in presence of a well-established allodynic and hyperalgesic state (day 7). As illustrated in panel b, 3 days of chronic PC1 treatment (day 11 VCR) are enough to abolish thermal allodynia (VCR vs VCR+ PC1, $p< 0.0001$) while it takes 7 days (day 14 VCR) to completely reverse mechanical allodynia (panel a, VCR vs VCR+ PC1, $p< 0.0001$). PC1 was also able

to significantly counteract hyperalgesia. Its effect is already evident at day 11 (3 days of PC1; VCR vs VCR +PC1 $p< 0.0001$) and is maintained until the end of PC1 schedule (day 14; VCR vs VCR +PC1 $p< 0.0001$). In this case paw withdrawal latency of PC1 treated mice were still different from those of CTR mice (panel c; CTR vs VCR+ PC1 $p< 0.0001$).

As shown in the same figure, VCR treated mice are not characterized by emotional alterations (panels d-i). At the end of VCR protocol (day 14) we did not detect in mice the presence of anxiety-like behavior. No alterations were observed between control (vehicle) and VCR treated mice in the time spent moving, evaluated in the open field test (panel d), in the novelty suppressed feeding test measuring both the latency for eating and the home-cage food consumption (panel e) nor in the number of buried marbles evaluated by marble burying test (panel f). Moreover, no signs of anxiety like behaviors were highlighted in the light/dark test (panel g), neither evaluating the time spent in the light box nor the number of transitions between the two chambers. In addition, VCR treated mice were similar to CTR mice considering the immobility time in the forced swim test (panel h) and the percentage of sucrose intake in the sucrose preference test (panel i), excluding the presence of a depressive like behavior in VCR treated mice.

3.2 Neuroinflammation assessment in DRG and spinal cord

Biochemical parameters were evaluated before starting PC1 treatment, corresponding to day 7 of VCR schedule protocol, and at the end of VCR/ VCR +PC1 treatment (day 14). All evaluations were performed in blind.

3.2.1 PK system (PK2 and PK-Rs) and neuroinflammation in DRG

As shown in figure 3, at day 7 an up-regulation of both PK-R1 (panel b; p= 0.0357) and PK-R2 (panel c; p= 0.0004) expression levels is already evident in DRG. At day 14, together with high levels of PK-Rs, we also registered increased levels of PK2 (panel a; p= 0.0157). PC1 treatment was able to prevent PK2 increase (panel a; VCR vs VCR+ PC1 p= 0.0067) and to reduce PK-Rs levels (VCR vs VCR + PC1 p= 0.0082 and p< 0.0001 panels b and c respectively). At day 7 we only observed changes in the levels of the proinflammatory cytokine IL-1β (panel d, p= 0.0156) without any change in the other cytokines (panels e-g). Simultaneously, we measured high levels of TLR4 (panel h, p= 0.0280) and of the marker of macrophage activation CD68 (panel i, p= 0.0260). These alterations are associated to the presence of increased levels of the marker of neuronal damage ATF3 (panel m, p= 0.0005). At the end of VCR protocol (day 14), we registered a significant neuroinflammatory condition characterized by a marked proinflammatory cytokine profile due to high levels of IL-1β, TNFα and IL-6 (panels d, e and f respectively; p= 0.001, 0.0059 and 0.0002) and low levels of the anti-inflammatory cytokine IL-10 (panel g, p= 0.0362). At the same time, TLR4, CD68 and ATF3 (panels h,i and m) were still upregulated and we also observed a significant increase of CD11b (panel l, p= 0.0103). PC1 treatment was able to counteract this neuroinflammatory condition, restoring a correct cytokine balance: reducing IL-1β levels (panel d, VCR vs VCR+ PC1 p< 0.0001), preventing TNFα and IL-6 increase (panels e and f, VCR vs VCR+ PC1 p= 0.0003 and p< 0.0001) and opposing to IL-10 decrease (panel g, p< 0.0001). Simultaneously the antagonist also normalized the levels of TLR4 (panel h, VCR vs VCR+ PC1 p= 0.0010), CD68 (panel i, VCR vs VCR+ PC1 p= 0.004) and

CD11b (panel l, VCR vs VCR+ PC1 p= 0.0236) even if it was unable to reduce ATF3 in a significant way (panel m).

SPINAL CORD

3.2.2 PK system (PK2 and PK-Rs), and neuroinflammation in spinal cord

Similarly to DRG, also in spinal cord (figure 4) at day 7 we only observed increased levels of PK-Rs, both PK-R1 (panel b, p= 0.0127) and PK-R2 (panel c, p=0.0208) without changes of PK2 (panel a). At the end of VCR protocol (day 14) we measured an up-regulation of the entire PK system (panels a and b: p= 0.0004, panel c: p= 0.0020). PC1 treatment was able to normalize the levels of all PK system members, opposing to PK2 upregulation (panel a, VCR vs VCR+PC1 p= 0.0002) and reducing PK-Rs levels (VCRvs VCR+PC1 p=0.0002 and p< 0.0001, panels b and c). As shown in the same figure after 7 days of VCR daily treatment we observed a significant upregulation of CD68 (panel i, p= 0.007) without any other alteration in cytokines levels (panels d-g), TLR4 (panel h), glial markers (l-m) or ATF3 (panel n). Differently, at the end of VCR protocol (day 14) we observed increased levels of the proinflammatory cytokines IL-1β and TNFα (p= 0.0146 and p= 0.0004; panels d and e respectively), without changes in IL-6 (panel f) and IL-10 (panel g), increased levels of TLR4 (panel h; p= 0.0383), CD68, CD11b (panels i and l respectively; p= 0.0001 and p= 0.0159), GFAP (panel m, p= 0.0001) and ATF3 (panel n, p= 0.0033). PC1 treatment could counteract and prevent neuroinflammation maintaining at control levels the mRNA levels of the cytokines IL-1β and TNFα (panels d and e; VCR vs VCR+PC1 p= 0.0007 and p= 0.0153), TLR4 and CD11b (panels h and l, p= 0.0322 and p= 0.0366) and reducing CD68 and ATF3 levels (panels i and n; p=

0.0016 and p< 0.0001). PC1 was unable to contrast the increase of GFAP observed at the higher VCR dose (panel m).

3.3 Evaluation of PK members and neuroinflammation in prefrontal cortex and hippocampus

Figure 5 shows changes in PK2 and PK-Rs and neuroinflammatory parameters evaluated at the end of VCR treatment (day 14) in prefrontal cortex (PFC) and hippocampus (HPC).

As shown in panels a and c, a decrease of PK2 (p= 0.0079) and PK-R2 receptor (p= 0.0225) in PFC of VCR treated mice was evident. PC1 could completely prevent the down regulation of PK-R2 (panel c; VCR vs VCR+PC1 p= 0.0264; CTR vs VCR+ PC1 ns). Similarly, we observed a down regulation of PK2 also in HPC (panel a; p= 0.0003) and PC1 treatment significantly prevented it (VCR vs VCR+PC1 p= 0.0002). As illustrated in the same figure, no significant alterations in the levels of pro/anti-inflammatory cytokines IL-1β (panel d), TNFα (panel e), IL-6 (panel f) and IL-10 (panel g) were detected in these two areas. However, we observed in the hippocampus a significant downregulation of CD68 (panel h; p= 0.0331), CD11b (panel i; p=0.0287) and GFAP (panel l; p< 0.0001) markers and PC1 treatment was able to prevent the GFAP decrease (panel l; VCR vs VCR+PC1 p= 0.0007).

4. Discussion

In this study we described a role of PK2 recently recognized as important mediator at cross roads between inflammation and pain (Franchi et al., 2017), in the progression of vincristine (VCR) -induced neuropathic pain. We also demonstrated that the antagonism of PK-Rs completely abolished allodynia in neuropathic mice. This effect was in part due to the ability of the PK-Rs antagonist, PC1, to contrast

neuroinflammation in dorsal root ganglia and to prevent its development in the spinal cord.

Chemotherapy-induced peripheral neuropathy is a frequent side effect of several antitumoral treatments, its development seems to be independent from drug's antitumoral mechanism of action and involves several components i.e. oxidative stress, mitochondrial damage, TRPV activation and neuroinflammation (Carozzi et al., 2015; Boyette-Davis et al., 2015). In particular, it was suggested a crucial role of activated macrophages and glial cells like microglia and astrocytes. These cells can release several proinflammatory cytokines and chemokines leading to neural excitability and pain transmission enhancement (Muthuraman et al., 2011; Montague et al., 2018; Brandolini et al., 2019). In our protocol (Kiguchi et al., 2008) VCR induced in mice a progressive mechanical and thermal allodynia as well as the development of thermal hyperalgesia. Our results suggest that after 7 days of chronic VCR treatment the presence of hypersensitivity is sustained by a neuroinflammatory condition particularly evident in DRG and not already manifest in the spinal cord. Therefore, our results confirmed that DRG represents one of the first direct target of the cytotoxic action of VCR, likely due to drug's capability to cross the blood nerve barrier (Hunt et al., 2012). In fact, probably as response to drug's toxic insult (Hunt et al., 2012; Hansen et al., 2011), we observed in DRG increased levels of the marker of neuronal damage ATF3, togheter with high levels of the marker of macrophage activation CD68, increased levels of TLR4, PK-Rs and IL-1β upregulation.Our data are in accordance with literature that describes a crucial role of TLR4 and of infiltered macrophages into PNS (Zhang et al., 2016; Montague and Malcangio 2017; Flatters et al., 2017); once activated these cells release a broad spectrum of proinflammatory mediators like cytokines and chemokines contributing

476 to damage and sensitizing process (Uçeyler et al, 2006; Lees et al., 2017; Montague
477 et al., 2018; Colvin 2019). At the end of VCR protocol, we registered in the animals a
478 further lowering of the mechanical and thermal thresholds that was related to a more
479 pronounced inflammatory profile at DRG level and to the development of a
480 neuroinflammatory condition clearly evident also in spinal cord. This condition is
481 sustained by an upregulation of the entire PK system (PK2 and PK-Rs) at both
482 peripheral and spinal cord level. Although we are aware that a limitation of our study
483 is that we did not perform an immunohistochemical analysis in the VCR model, we
484 have deeply investigated the cells associated to PK system in different models of
485 neuropathic pain in our previous works (Maftei et al., 2014; Guida et al., 2015)
486 including (Moschetti et al., 2019) in bortezomib induced peripheral neuropathy.

487 Considering that infiltrating macrophages express PK-Rs receptors (LeCouter et al.,
488 2004), PK2 can act in autocrine or paracrine way sustaining a neuroinflammatory
489 loop that exacerbate the neuronal damage and promote a progressive glial activation
490 at the spinal cord level. However, as previously described, other cell types like
491 satellite glial cells, neurons and astrocytes express PK-Rs and are important source
492 of PK2 (Maftei et al., 2014; Guida et al., 2015; Moschetti et al., 2019). We could
493 speculate that the proinflammatory cytokine increase in DRG can trigger the
494 activation of the transcriptional factor STAT3, which in turn directly binds to the Pk2
495 promoter (Qu et al. 2012) inducing PK2 increase. On the basis of our results we can
496 suppose that PK2, differently from the chemokine CX3CR1 (Montague et al., 2018),
497 is not directly involved in the initial recruitment phase of immune cells in the
498 damaged tissue, and it is not related to the onset of hypersensitivity, as has been
499 observed in other neuropathic pain models (Maftei et al., 2014; Guida et al., 2015;
500 Castelli et al., 2016), In this experimethal model, as already described for bortezomib

(Moschetti et al., 2019), PK2 has a role in the progression and worsening of a neuroinflammatory and hypersensitivity state, sustaining a flow of neuroimmune activation from the periphery to the central nervous system (Scholz and Woolf, 2007; Kiguchi et al., 2009; Calvo et al., 2012; Sacerdote et al., 2013) which is at the basis of the development of pathological pain. Chronic therapeutic treatment with PC1 can block the already started, although still at the beginning, neuroinflammatory process preventing PK2 upregulation and contrasting a self-sustained inflammatory/ neuroinflammatory loop that involves also cytokines. The result of this condition is a complete anti-allodynic effect exerted by PC1 and a partial anti-hyperalgesic one. As happened in bortezomib induced neuropathy, PC1 fails to counteract the increase of GFAP in the spinal cord, so we believe that astrogliosis might be responsible of the only partial anti-hyperalgesic effect of PC1 (Moschetti et al., 2019). We are aware that we did not provide a resolutive evidence that the effects of PC1 on vincristine-induced pain are only due to its interactions with PK-Rs but several data can support this hypotheis. In particular, when administered alone in healthy mice PC1 did not induce behavioural/biochemical alterations (Castelli et al.2016; Giannini et al., 2009) and this antagonist did not counteract hypersensitivity induced by molecules different from PK2, i.e. PGE2, ATP or bradykinin. Other PK-Rs peptidic or non peptidic antagonsists exert anti-inflammatory and modulatory effects similar to those induced by PC1 (Abou-Hamdan et al, 2015; Maftei et al., 2014) and different strategies that block PK2 activity, such as antibodies against PK2 prevent pain and neuroinflammation in a model of cancer pain (Hang et al., 2015). All these results seem to indicate that PC1 is effective in blocking PK2 activity, exogenously administered or endogenously overexpressed.

The results here obtained assume a broader meaning because, confirming our recently published data on bortezomib-induced neuropathy (Moschetti et al., 2019), suggest that the block of PK system counteracts the activation of intracellular signaling pathways common to antitumoral drugs characterized by different primary mechanism of action and responsible for neuropathy development.

Both chronic pain and chemotherapeutics are often, but not always, associated to altered emotional behavior and impaired cognitive function (Moriarty et al., 2011; Bushnell et al., 2013; Seigers et al., 2013; Palazzo et al., 2016; Santos and Pyter, 2018; Guimaraes et al., 2018). However some papers suggest a temporal shift between the presence of the symptoms and the appearance of altered affective behavior (Dellarole et al., 2014 ; Toma et al., 2017). Also in our experimental paradigm, in VCR treated animals, pain is not correlated to the presence of any anxiety or depressive like behavior. Our results demonstrate that 2 weeks of continuous marked thermal and mechanical hypersensitivity are not associated to overt mood alteration, suggesting that development of mood/cognitive alteration may vary according to protocols, timing and models utilized (Karl et al., 2019).

Considering that chemotherapeutics do not or only partially cross the blood brain barrier, central nervous system has generally been considered to be less vulnerable to a direct toxic effect of chemotherapy. However, it was suggested that chemotherapy metabolites or other indirect mechanisms, such as peripheral immune cell infiltration may have neurobiological consequences (Ahles and Saykin, 2007). In our experimental protocol we did not find emotional-like deficits in VCR treated animals and, at the same time, we did not observe changes in the levels of proinflammatory cytokines in brain areas generally involved in mood regulation like prefrontal cortex and hippocampus. These data are in accordance with literature that

suggests a crucial role of neuroactive cytokines like IL-1β, IL-6 and TNFα in the development of a depressive symptomatology (Dantzer 2018; Pfau et al., 2018; Bhattacharya and Jones, 2018). It was also reported a role of the same PK2 in mood regulation: intracerebroventricular injection of PK2 leads to increase of anxiety and depressive like behavior while mice deficient in PK2 displayed reduced anxiety and depression (Li et al., 2009). At our evaluation time we observed low levels of PK2 in the same supraspinal areas and in HPC we also observed a downregulation of glial markers. This condition, probably due to an initial loss of glial cells, suggests the onset of a neuroinflammatory/ neurotoxic condition (Czèh et al., 2006; Banasr et al., 2010) in brain tissues. PC1 treatment can counteract this process by normalizing GFAP and PK2 levels in HPC, suggesting that astrocytes may be important producers of PK2 not only in spinal cord (Maftei et al., 2014; Moschetti et al., 2019), but also in the hippocampus. In a recent pre-clinical study on Paclitaxel (Toma et al., 2017) it was detected a temporal shift between the onset of pain and emotional-like deficits. On the basis of these results, we can not exclude that the precocious biochemichal alterations observed in brain tissues could lead to the development of altered affective like behavior at longer evaluation times or in presence of multiple cycles of VCR.

In conclusion this study described a role of PK2 in vincristine-induced neuropathic pain and suggests that the antagonism of PK system could represent a common strategic target to manage chemotherapy-induced neuropathic pain counteracting the development and progression of a neuroinflammatory condition.

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Legends

FIGURE 1: Timeline representing the experimental design.

VCR: Vincristine sulfate

PC1: PK-Rs antagonist

FIGURE 2: Effect of VCR treatment on hypersensitivity (allodynia and hyperalgesia) and affective behavior (anxiety and depressive like behavior). Effect of PK-Rs antagonist (PC1) on hypersensitivity.

a-c: Panels a, b and c show the time course development of mechanical (panel a) and thermal (panel b) allodynia as well as thermal hyperalgesia (panel c) in mice chronically treated with vincristine for 14 consecutive days (VCR; 0.1mg/ kg, once a day). In the same panels it is reported the effect of PC1 treatment. PC1 was administered (s.c. 150µg/kg twice a day) for 7 days starting from day 7 (established hypersensitivity) until the end of VCR schedule, day 14. Data are presented as mean±SEM of 18 mice/group. Statistical analysis was performed by mean of Two way-ANOVA analysis of variance with repeated measures followed by Bonferroni's post test. Treatment: $F(2, 51)$, panel a= 296.9 ; panel b= 75.18; panel c= 424. 8, $p<0.0001$. Time: $F(4, 204)$ panel a=165.9, panel b=46.19, panel c=121.8, $p<0.0001$. Interaction: $F(8, 204)$ panel a= 105.8, panel b=18.73, panel c=41.65, $p<0.0001$. Bonferroni post test: ***$p<0.001$ vs CTR (saline treated mice); °°° $p< 0.001$ vs VCR; $^{++}p<0.01$, $^{+++}p<0.001$ vs VCR mice at the previous time point. **d-i:** Panels d, e, f and g show the effect of 14 days of VCR treatment on anxiety-like behavior, evaluated in an anxiety like cohort of animals, by means of open field (panel d), novelty suppressed feeding (panel e), marble burying (panel f) and dark/light box test (panel g). At the same time (day 14) depressive-like behavior was tested in a depression-like cohort of mice by using the forced swim test (panel

h) and sucrose preference test (panel i). Data are presented as mean± SEM of 8 animals per group. Statistical analysis was performed by means of two-tailed unpaired T-test.

FIGURE 3: PK system and neuroinflammatory markers in DRG.

mRNA levels of PK system members and of neuroinflammatory markers were measured in DRG by using Real Time-qPCR. Evaluations were performed after 7 days of chronic VCR administration in CTR and VCR mice and at the end of the chemotherapeutic schedule (day 14) in CTR, VCR and VCR + PC1 mice. Results 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 from 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. **a-c:** Panels a, b and c report the mRNA levels of PK2, PK-R1 and PK-R2 respectively. 7 days: PK2: t=1.21, p=0.26; PK-R1: t=2.616, p=0.0357; PK-R2: t=4.894, p=0.0004. Day 14:PK2: $F_{(2,13)}$=8.482, p=0.0044; PK-R1: $F_{(2,12)=}$8.715, p=0.0046; PK-R2: $F_{(2,21)}$ =29.17, p<0.0001. **d-g:** Panels d, e and f represent respectively the mRNA levels of the proinflammatory cytokines IL-1β (7 days, t=2.995, p=0.0156; 14 days $F_{(2,12)}$ =24.16, p<0.0001), TNF-α (7 days, t=0.034, p=0.97; 14 days $F_{(2,12)}$ =17.27,p=0.0003) and IL-6 (7 days, t=0.18, p=0.86; 14 days $F_{(2,12)}$ =25.85 , p<0.0001) while panel g reports that of the anti-inflammatory cytokine IL-10 (7 days, t=1.28, p=0.237; 14 days $F_{(2,15)}$ =28.09, p<0.0001. **h-m:** mRNA levels of TLR4 (panel h, 7 days, t=2.7, p=0.028; 14 days $F_{(2,22)}$ =11.19, p=0.0004), CD68 (panel i, 7 days, t=2.744, p=0.026; 14 days $F_{(2,21)}$ =10.66, p=0.0006) and CD11b (panel l, 7 days, t=0.745, p=0.478; 14 days $F_{(2,14)}$ =7.196, p=0.0071) and of the marker of neuronal damage ATF3 (panel m, 7 days, t=5.35, p=0.0005; 14 days $F_{(2,15)}$ =6.897, p=0.0075). Bonferroni 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, °°°p<0.001 vs VCR day 14.

FIGURE 4: PK system and neuroinflammatory markers in spinal cord.

mRNA levels of PK system members and of neuroinflammatory markers were measured in spinal cord by using Real Time-qPCR. Evaluations were performed after 7 days of chronic VCR administration in CTR and VCR mice and at the end of the chemotherapeutic schedule (day 14) in CTR, VCR and VCR + PC1mice. Data are presented as mean±SEM from 5-8 mice per group. At day 7, statistical analysis was performed by means of two tails unpaired T-test while at day 14 by One way-Anova analysis of variance followed by Bonferroni's post test. **a-c:** Panels a, b and c report the mRNA levels of PK2, PK-R1 and PK-R2 respectively. 7 days: PK2: t=1.938, p=0.108; PK-R1: t=4.227, p=0.0127; PK-R2: t=3.685, p=0.021. Day 14: PK2: $F_{(2,15)}$ =18.82, p<0.0001; PK-R1: $F_{(2,12)}$ =21.7, p=0.0001; PK-R2: $F_{(2,12)}$=27, p<0.0001. **d-g:** Panels d, e and f represent respectively the mRNA levels of the proinflammatory cytokines IL-1β (7 days, t=0.661, p=0.526; 14 days $F_{(2,21)}$ =10.44,p=0.0007), TNF-α (7 days, t=0.252, p=0.808; 14 days $F_{(2,21)}$ =11.12, p=0.0005), and IL-6 (7 days, t=1.633, p=0.17; 14 days $F_{(2,12)}$ =1.804, p=0.206), while panel g reports that of the anti-inflammatory cytokine IL-10 (7 days, t=0.0427, p=0.967; 14 days $F_{(2,15)}$ =0.0814, p=0.92). **h-n:** mRNA levels of TLR4 (panel h, 7 days, t=0.885, p=0.412; 14 days $F_{(2,12)}$ =5.886, p=0.0165), of the glial markers: CD68 (panel i, 7 days, t=3.632, p=0.007; 14 days $F_{(2,12)}$ =21.52, p=0.0001), CD11b (panel l, 7 days, t=1.234, p=0.279; 14 days $F_{(2,12)}$ =6.808, p=0.011) and GFAP (panel m, 7 days, t=0.520, p=0.618; 14 days $F_{(2,12)}$ =28.6, p<0.0001) and of the marker of neuronal damage ATF3 (panel n, 7 days, t= 1.924, p=0.122; 14 days $F_{(2,12)}$ =26.13, p<0.0001). Bonferroni's post: *p<0.05, **p<0.01, ***p<0.001 vs CTR (at the same time point); °p<0.05, °°p< 0.01, °°°p<0.001 vs VCR day 14.

FIGURE 5: PK system and neuroinflammatory markers in prefrontal cortex and hippocampus.

mRNA levels of PK system members and of neuroinflammatory markers measured at the end of the chemotherapeutic schedule (day 14) in prefrontal cortex (PFC) and hippocampus (HPC) of CTR, VCR and VCR + PC1 mice by using Real Time-qPCR. Results were expressed in relation to GAPDH and presented as fold-increases over the levels of CTR animals. Data are presented as mean±SEM of 5 mice/ group. Statistical analysis were performed using One way-Anova analysis of variance followed by Bonferroni's post test. **a-c:** Panels a, b and c report the mRNA levels of PK2 (PFC: $F_{(2,12)}$ =7.198, p=0.0088; HPC: $F_{(2,12)}$ =22.36, p<0.0001), PK-R1 (PFC: $F_{(2,12)}$ =3.406, p=0.067; HPC: $F_{(2,12)}$ =2.23, p=0.15) and PK-R2 (PFC: $F_{(2,12)}$ =6.688, p=0.011; HPC: $F_{(2,12)}$ =0.787, p=0.478) respectively. **d-g:** mRNA levels of the proinflammatory cytokines IL-1β (d), TNF-α (e) and IL-6 (f) and of the anti-inflammatory cytokine IL-10 (g). **h-l:** mRNA levels of the glial markers: CD68 (panel h, PFC: $F_{(2,12)}$ =0.997, p=0.398; HPC: $F_{(2,12)}$ =4.68, p=0.03), CD11b (panel i, PFC: $F_{(2,12)}$ =2.954, p=0.0906; HPC: $F_{(2,12)}$ =5.371, p=0.022) and GFAP (panel l, PFC: $F_{(2,12)}$=0.455, p=0.645; HPC: $F_{(2,12)}$ =23.69, p<0.0001). Bonferroni post test: *p<0.05, **p<0.01 vs CTR; °p<0.05, °°p<0.01 vs VCR.