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CONTROLLING THE ACTIVATION OF THE PROKINETICIN SYSTEM AS THERAPEUTIC APPROACH TO RELIEF NEUROPATHIC PAIN AND REDUCE NEUROINFLAMMATION

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SUMMARY

Neuropathic pain is a relevant clinical problem worldwide, since current therapeutic treatments are unsatisfactory. The identification of novel therapeutic targets and the development of new pharmacological approaches remain a priority. This pathological condition is generally triggered by an injury at peripheral or central nervous system and it is characterized by pain exacerbation and neuronal hypersensitization, resulting in abnormal pain transmission. Neuroinflammation in the peripheral and central nervous system largely contributes to neuropathic pain onset, development and maintenance. In this scenario, the recently identified chemokine family, the prokineticin system (PKS), is a promising pharmacological target for the management of neuropathic pain, considering its pronociceptive and proinflammatory properties and its role in neuronal-glia interaction. Moreover, the availability of specific receptor antagonists makes this system even more interesting in order to control prokineticin activity. In this review we report all preclinical data available on the role of PKS in the physiopathology of neuropathic pain. The results clearly suggest that drugs which block the PKS may represent an innovative and efficacious pharmacological treatment to control neuropathic pain in patients.

Key words
Prokineticin system;
neuropathic pain;
neuroinflammation;
animal models.

Impact statement

- PK2/PKRs play a pivotal role in pain transmission.
- Neuropathic pain state increases PK2/PKRs levels in the main pain stations.
- Blocking PKRs with specific antagonists reduces pain and neuroinflammation.
- Prokineticin system opens a new therapeutic avenue for neuropathic pain treatment.

Abbreviations

PNS: peripheral nervous system; CNS: central nervous system; PKS: prokineticin system; PK1: prokineticin 1; PK2: prokineticin 2; PKs: prokineticins; PKR1: prokineticin receptor 1; PKR2: prokineticin receptor 2; PKRs: prokineticin receptors; EG-VEGF: vascular endothelial factor of the endocrine gland; aa: amino acids; Trp: Tryptophan; NPY: Neuropeptide Y; cAMP: Adenosine monophosphate cyclique; MAPK: Mitogen-activated protein kinase; Ala: Alanine; val: valine; TRPV1: transient receptor potential vanilloid receptor 1; CFA: Freund's Complete Adjuvant; hr: hour; DRG: dorsal root ganglia; WT: Wild Type; TRPA1: Transient receptor potential ankyrin 1; CGRP: calcitonin gene-related peptide; SP: Substance P; CCI: constriction nerve injury; SCI: spared nerve injury; CIBP: cancer-induced bone pain; STZ: streptozotocin; VCR: vincristine; BTZ: bortezomib; c.d.: cumulative dose; PWT: paw withdrawal threshold; PWL: paw withdrawal latency; sc: subcutaneous; iv: intravenous; PN: perineural; IT: intrathecal; min: minutes; PAG: periaqueductal gray; IL-1β: Interleukin 1 beta; IL-10: Interleukin 10; CD11b: Cluster Of Differentiation 11b; CD68: Cluster Of Differentiation 68; TLR4: Toll-like receptor 4; IL-6: Interleukin 6; TNFα: Tumour Necrosis Factor alpha; GFAP: Glial Fibrillary Acidic Protein; ATF3: Activating transcription factor 3; CD206: Cluster of Differentiation 206; iba-1: Allograft inflammatory factor 1; KDM6A: Lysine-specific demethylase 6A; PPAR: Peroxisome proliferator-activated receptor; DRG: dorsal root ganglia; PFC: prefrontal cortex; HPC: hippocampus; HPT: hypothalamus.

INTRODUCTION

As well known, acute pain has a physiologically protective role, since it warns body about an ongoing or impending tissue damage, in order to elicit appropriate behavioral responses to minimize it. When tissue damage occurs, there are changes in excitability of peripheral and central nervous system (PNS and CNS), which transmit nociceptive information from the site where the *noxa* is present up to the cortex. In the inflamed tissue sustained but reversible hypersensitivity may occur and the triggering of these mechanisms helps in the recovery process of wounds, thus avoiding any contact with the injured area until healing. In contrast, chronic pain does not offer biological or adaptive advantages, and becomes a disease itself. In particular, neuropathic pain is a highly debilitating form of chronic pain generally triggered by direct or indirect injury at PNS or CNS level and is one of the most important clinical problems worldwide (1). This pathological condition is characterized by pain exacerbation (in particular with allodynia and hyperalgesia development) and neuronal hypersensitization at spinal and supraspinal level, which lead to an abnormal pain transmission (2). The cause of neuropathic pain development cannot be always established or reversed (3). Indeed, its pathophysiology is very complex: imbalances between excitatory and inhibitory somatosensory signaling, ion channels alterations and abnormal immune reactions, associated with neuronal and synaptic plasticity, are all implicated in neuropathic pain states (4-6). Emerging evidence indicate that neuronal activity enhancement requires glial cells activation. These cells are physiologically involved in homeostasis maintaining, supporting and protecting neuronal cells (7), however, in pathological conditions, such as during a chronic pain condition, they become activated, proliferate, change their morphology and release pro-inflammatory mediators that promote neuronal sensitization (8-10).

It is now evident that pro- and anti- inflammatory cytokines produced by resident and infiltrating immune cells in the nervous system and by glial cells are common denominators in neuropathic pain (11). Indeed, cytokines start a cascade of events related to neuroinflammation which can maintain and/or worsen the original lesion, contributing to pain generation and its chronicization (12). In addition, current therapeutic tools are unsatisfactory since this type of pain is frequently resistant to available treatments (13, 14). For these reasons, the identification of novel therapeutic targets and the development of new pharmacological approaches for neuropathic pain remain a challenge. In this scenario, a novel class of chemokines and their receptors, the prokineticin system (PKS) have recently been demonstrated to have an important role in neuropathic pain, sustaining pain and neuroinflammation and appear to be a promising pharmacological target for the management of this type of pain.

MATERIALS AND METHODS

The literature research was conducted between November and December 2021 via the PubMed, EMBASE and Cochrane Library databases. No filter time was used and only papers in English language were considered. Key terms used were 'neuropathic pain' OR 'neuropathy' AND 'prokineticin system' OR 'prokineticins' OR 'prokineticin antagonism' and were searched in paper title, abstract and keywords.

All titles and abstracts were independently revised by two authors (GA and DM) to assess their relevance for the inclusion in this review. In addition, some publications were searched in articles/ reviews reference lists on this topic and key publications were also identified through searches in the authors' files.

Full texts of manuscripts/reviews were analyzed by authors and 52 papers were included in this review.

PROKINETICIN SYSTEM

The prokineticin system, a new family of chemokines identified in 2001, includes two mammalian proteins, prokineticin 1 and prokineticin 2 (PK1 and PK2, respectively) and their receptors, PKR1 and PKR2. PK2 is also known as BV8 and was first isolated from the skin of the frog *Bombina variegata*, while PK1 is also known as *endocrine gland-derived vascular endothelial growth factor* (EG-VEGF). Homologous and orthologous of prokineticins (PKs) are highly conserved across species, indeed prokineticin-like peptides are present in invertebrates, *i.e.* shrimp and crayfish; vertebrates *i.e.* frog, black mamba snake, fugu and trout; and mammals *i.e.* bull, rodents, monkey and humans (15, 16). PK1 and PK2 are bioactive peptides of about 10 kDa with regulatory activity and consist of 86 and 81 amino acids, respectively. PKs share approximately 44% amino acid identity. Both chemokines have a structurally conserved motif characterized by a carboxyl-terminal cysteine-rich domain that forms five disulfide bridges with conserved spacing, a Trp residue in position 24 and an N-terminal AVITGA sequences, which is essential for the correct binding of receptors. These highly conserved homologies among species have been shown to be indispensable for the bioactivity of PKs (17, 18). The PK receptors (PKRs) have been identified in humans, rats and mice and are G protein coupled receptors (19-21). PKs can bind and activate both receptors. However, the signal transduction efficacy of PKR1 is slightly higher than the one of PKR2. It has been shown that activation of PKRs leads to accumulation of inositol phosphate and mobilization of intracellular Ca^{2+} via $G_{q/G11}$ proteins. In addition, PKRs may stimulate or inhibit cAMP accumulation through G_s or G_i proteins, respectively. Furthermore, PKRs can stimulate MAPK (mitogen-activated protein kinase) via G_o protein-mediated signaling (17, 22). PKs and their receptors are widely expressed in several organs and tissues. In particular PKs are co-expressed in brain, spinal cord, dorsal root ganglia, ovary, placenta, prostate, testis, adrenal cortex, peripheral blood cells, intestinal tract, spleen, pancreas, heart and bone marrow. However, there are also some differences; indeed, PK1 is predominantly expressed in steroidogenic organs, whereas PK2 is primarily, but not exclusively, expressed in the central nervous system and immune cells (23, 24). Besides, PKRs are co-expressed in certain tissues, but while PRR1 is mainly expressed in peripheral tissues, PKR2 results abundantly expressed in the brain (17, 25). Both receptors, however, are co-expressed also in small and medium-sized DRG cells as well as in the spinal cord. PKS has been linked to several biological effects like intestinal motility, neurogenesis, angiogenesis, circadian rhythms, haematopoiesis and nociception. Emerging evidence have also indicated its involvement in pathologies which affect nervous and reproductive systems, myocardial infarction and tumorigenesis. Moreover, PKS is also involved in sensory processing and nociceptive signalling and is an important player in inflammation and pain pathophysiology (24).

PROKINETICIN SYSTEM IN NOCICEPTION REGULATION

The first evidence of a pronociceptive role of PKS was reported by Negri and colleagues (26). In rodents, the injection of Bv8/ PK2 induced mechanical and thermal hyperalgesia (26). The local injection of a very low dose of Bv8 (50 fmol) into the paw decreased the nociceptive threshold which reaches its maximum in 1 hr and disappears in 2-3 hrs. Systemic injection (subcutaneous, sc, and intravenous, iv) of higher doses induced hyperalgesia with a characteristic biphasic trend: the first peak occurs in 1 hr and the second peak in 4-5 hrs. This suggests that the first one depends on a direct action on nociceptors while the second may depend on central and/or peripheral sensitization. Indeed, subsequent studies supported the physiological role of PKS as peripheral and central pain modulator. Mice lacking PKR (pkr-/-) or PK2 (pk2-/-) are less sensitive to noxious stimuli than wild-type (WT), showing impaired hyperalgesia development after tissue damage (27-30). In particular pk2-/- mice showed a strong reduction in nociception induced by thermal and chemical stimuli, indicating an important role for endogenous PK2 in pain sensitization (27). Although both PKR1 and PKR2 are expressed in superficial layers of spinal cord, DRGs and peripheral terminus of nociceptors, and both mice lacking of PKR1 or PKR2 are less sensitive than WT-mice to Bv8-induced heat hyperalgesia, highlighting a positive interaction between PKR1 and TRPV1 channel, only Pkr1-/- mice showed also impaired responsiveness to tactile allodynia (28, 30), whereas pkr2-/- mice showed reduced nociceptive response to cold temperature (4 °C), suggesting a functional interaction between PKR2 and TRPA1 channels (30). Moreover, the molecular mechanisms of Bv8-induced hyperalgesia have also been studied *in vitro*, in neurons of DRG primary cultures (30, 31). It was observed that the number of neurons responding to Bv8 stimulus through an increase of intracellular calcium was five times lower in Pkr1-/- mice than in WT mice (28). Furthermore, it was also demonstrated that the percentage of DRGs neurons Bv8-responsive which were also responsive to mustard oil, was much higher in pkr1-/- mice than in pkr2-/- mice and a high degree of co-localization of PKR1 and of the vanilloid receptors TRPV1 and TRPA1, has been found. Therefore, taken together, these findings suggest a functional interaction between PKRs and TRP channels in the development of hyperalgesia. Additionally, half of neurons that responded to Bv8 stimulus also expressed/ released neuropeptides such as CGRP (calcitonin gene-related peptide) and SP (Substance P) (32, 33). In addition, Bv8 microinjection into the PAG exerted a pronociceptive effect by increasing the intrinsic GABAergic tone which is responsible for the inhibition of the antinociceptive output of the neurons of PAG (34).

These *in vivo* studies demonstrated the involvement and the ability of PKS to modulate the central pain pathways.

ANTAGONISTS OF PROKINETICIN SYSTEM

Being the PKS involved in the regulation of a wide spectrum of biological functions and pathological conditions, the development of effective PKRs antagonists may be useful in the treatment of different pathological conditions. The antagonism of PKS signalling emerges also as a new promising approach to control different types of pain. The identification of structural determinants, necessary for both receptor binding and PKs activity, was fundamental to design functional PKR antagonists (35). Specifically, the highly conserved amino terminal sequence AVITGA and the Trp residue in position 24 are essential. As suggested by Miele and colleagues (36), AVIT proteins could interact with PKRs by orienting the protein region, including AVITGA sequence and the conserved Trp24. Moreover, it has also been demonstrated that deletions and/or substitutions in these conserved residues are able to produce antagonist molecules (37, 38). In addition, in Bv8 molecule the N-terminal deletion of the first two amino acids (Ala e Val) produces an analogue without biological activity but still capable to bind the receptors (named dAV-Bv8): in this way it acts as PKRs antagonist *in vitro* and *in vivo* (38). Even the substitution of Trp with Ala in position 24 produces antagonist-like protein (peptidic antagonist named A-24) (39). Unfortunately, the large size of these peptides makes their therapeutic use difficult and expensive. New promising non-peptidic PKR antagonists, triazine-guanidine derivates, have been synthetized and developed, *i.e.* PC1, PC7, PC10, PC18, PC25 and PC35 (**figure 1**) (35, 40). The different PC antagonists have been used in order to block the PKS activity in several pathological conditions. However only PC1 and PC7 were used in preclinical model of chronic pain. The "lead compound" is PC1. Indeed, PC1 mimics the structural features required for PKRs 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 (35). Results from binding assay demonstrated that PC1 is a ligand that binds both PKR1 and PKR2, although it prefers PKR1. *In vitro* studies revealed a clear antagonist activity of PC1 that was able to block Bv8-induced intracellular calcium increase in CHO cells transfected with PKR1 or

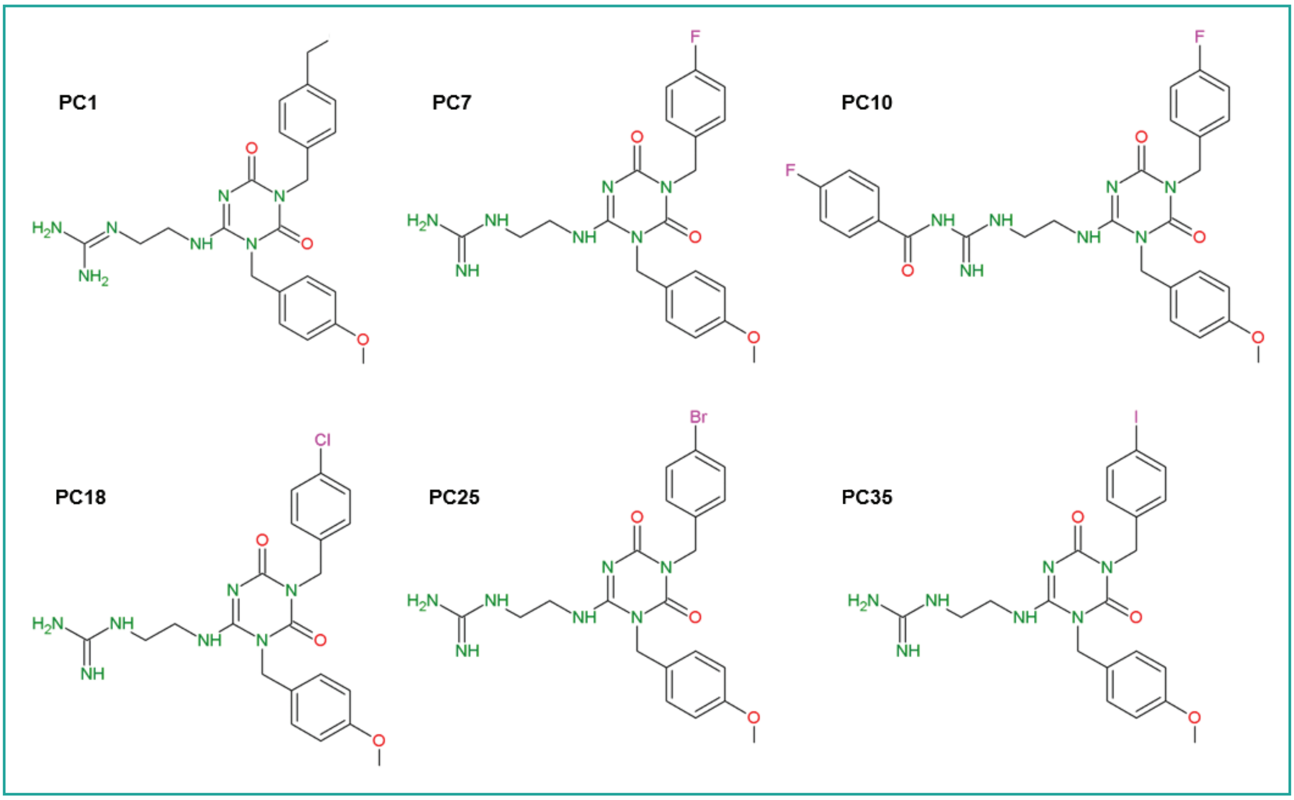


Figure 1. PK antagonists; triazine compounds.
2D chemical structure of synthetic organic compounds PC1, PC 7, PC10, PC18, PC25 and PC35.

PKR2 (35). Besides, *in vivo* studies demonstrated that both PC1 and PC7 were able to selectively antagonize Bv8-induced hyperalgesia, even if PC7 antagonizes it at doses ten times lower than PC1 (41). Moreover, PC1 also contrasts capsaicin-induced thermal hypersensitivity, suggesting that it may prevent the activation of PKRs and TRPV1 by their endogenous ligands (42, 43). In CFA-induced inflammatory pain model, systemic injections of PC1 (from 20 to 150 µg/kg, sc) reduced hyperalgesia in a dose-dependent manner, completely abolishing it at the dose of 150 µg/kg (44).

Besides these receptor antagonists, anti-Bv8 neutralizing antibodies are also commercially available, effectively capable of inhibiting PKS (45).

PROKINETICIN SYSTEM AND NEUROPATHIC PAIN

Studies aimed at identifying the link between PKS and neuropathic pain began in 2014. To date, 11 original manuscripts have been produced, and this review will illustrate the discoveries achieved so far. Neuropathic pain arises from both PNS and CNS lesions and many etiologies have been recognized in human. Several animal models of neuropathic pain, that mimic the different human conditions, are available and have been used to identify the role of PKs. Chronic constriction injury (CCI-model) (41, 46) and spared nerve injury (SCI-model) (47) mimic a direct nerve trauma and are the most frequently used. Painful neuropathy is a frequent complication of diabetes and STZ model (streptozotocin-induced diabetic neuropathy) represents the most commonly used model for the study of this type of pain (48). Peripheral neuropathy is a very frequent and severe side effect of chemotherapy and is often the limiting factor for achieving the effective dose; for this reason, a series of studies investigated the role of PKS in peripheral neuropathy induced by the chemotherapeutic vincristine (VCR-model) (49) and bortezomib (BTZ-model) (50-52). Moreover, a neuropathic pain component is often present also in cancer pain, and this aspect has been addressed in a model of cancer-induced bone pain (CIBP-model) (45). These studies were performed in male mice of the strain CD1 (41, 46, 47) or C57BL/6J (48-52) except the CIBP model which was induced in female Sprague-Dawley rats (45). All these models, develop a significant hypersensitivity to mechanical and/or thermal stimuli with a different temporal development. In particular, CCI-model is characterized by a decrease in paw withdrawal threshold and latency (PWT and PWL) as early as 3 days after sciatic nerve ligation (41, 46). After 5 days of induction, SCI model develops allodynia and hyperalgesia (47). The CIBP-model shows a gradual decrease in PWT from day 6 after tumour cell inoculation (45). Moderately low doses of STZ induced an evident mechanical allodynia starting from 14 days after treatment (48). Finally, both VCR and BTZ compounds induced a progressive development of mechanical and thermal allodynia, as well as of thermal hyperalgesia respectively 3 and 7 days after the first chemotherapic treatment (49-52).

Dose-finding experiments for PK antagonists in neuropathic pain

In a first series of studies, in order to identify the dose and the best route of administration for PKS antagonists, Negri's group performed a dose finding using PC1 (46) and PC7 (41) in the CCI-model and

data are reported in **figure 2**. Three days after CCI, in an evident state of hypersensitivity, mice were treated subcutaneously with 3 different doses of PC1 (30, 75 and 150 µg/kg) or PC7 (5, 15, 45 µg/kg). A single bolus of PC1 or PC7 reduced thermal hyperalgesia in a dose-dependent manner. The higher dose of both triazine compounds (PC1: 150 µg/kg and PC7: 45 µg/kg) restored thermal thresholds of pathological animals to basal level. This effect

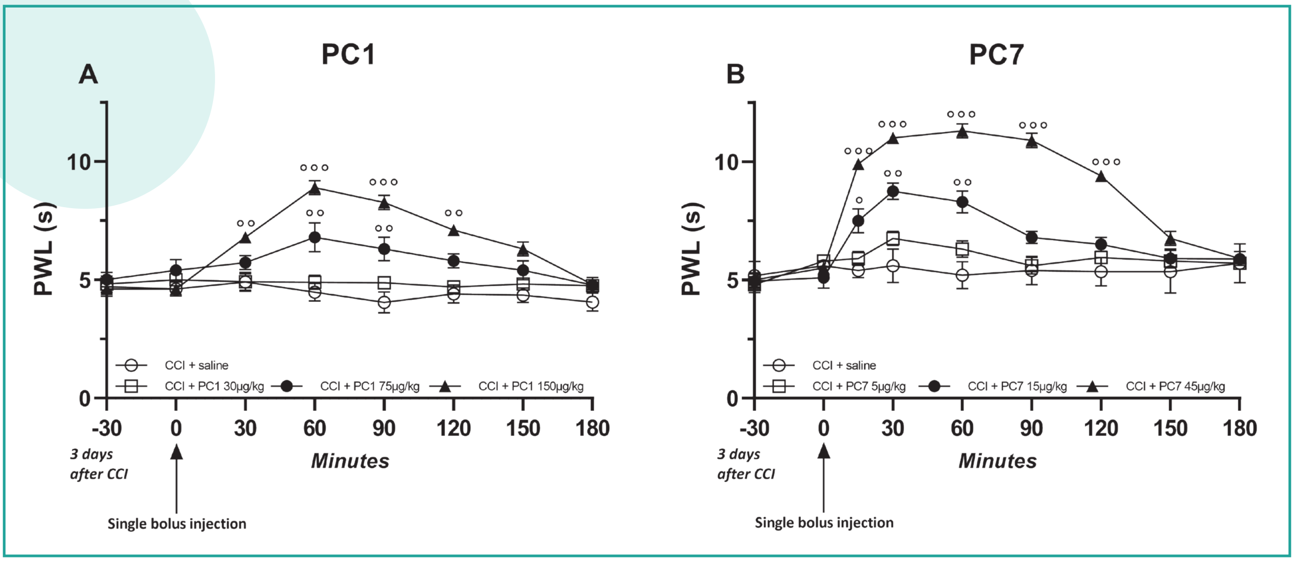


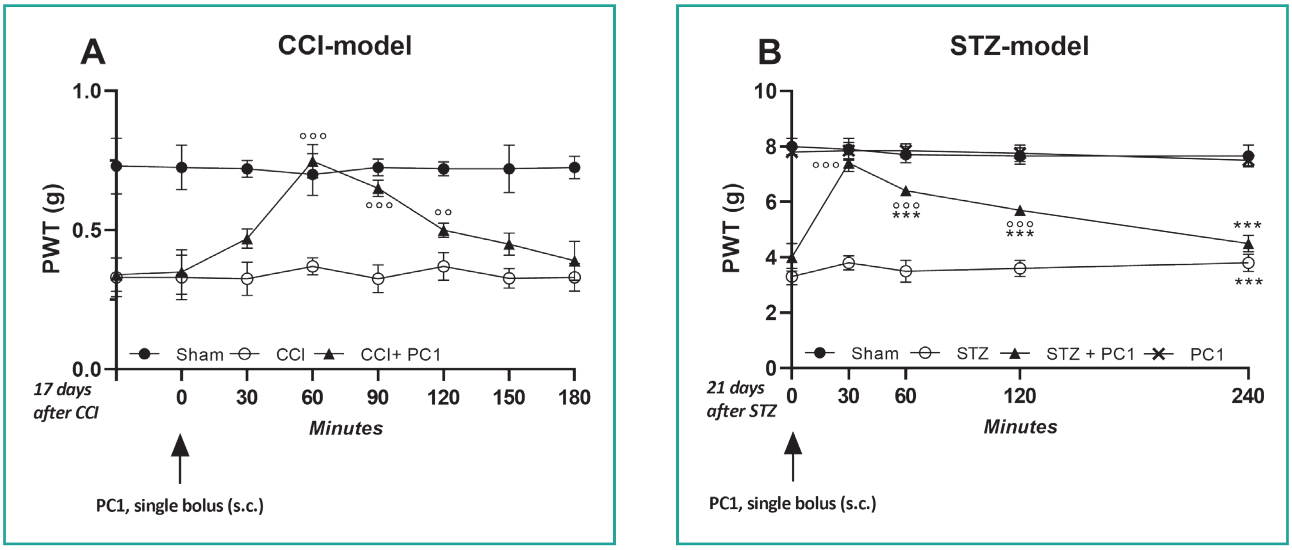
Figure 2. Dose-finding of PC1 and PC7.
A single bolus subcutaneous injection of PC1 (A, modified by Maftei *et al*., 2014) and PC7 (B, modified by Lattanzi *et al*., 2015) on day 3 after CCI restored the CCI-induced thermal hyperalgesia in a dose-dependent manner. PWL: paw withdrawal latency. The data represent the means ± SEM of 5 mice/group. Two-way ANOVA was used for statistical evaluation, followed by the Bonferroni's test. °$p < 0.05$; °°$p < 0.01$; °°°$p < 0.001$ *vs* CCI + saline mice.

remained statistically significant for 30-120 min after treatment.

Maftei and colleagues (46) also tested PC1 at 3 different doses (5, 15 and 50 ng) using two other different routes of administration, *i.e.* perineural (PN) and intrathecal (IT). Also in this case, regardless the route of administration, the higher dosage was the most effective, exerting an effect comparable to that of 150 µg/kg of PC1 injected subcutaneously. Since the subcutaneous route is faster, simpler and less stressful for animals than the perineural and intrathecal ones, in all subsequent studies where PKS was antagonized with PC1, the route of administration used was the subcutaneous one at the dose of 150 µg/kg. In all protocols PC1 was administered twice a day (41, 46-52).

PKS antagonism effect on hyperalgesia and allodynia

The acute effect of PKS antagonism was evaluated (**figure 3**) in CCI-, STZ- and BTZ-models of neuropathic pain (46, 48, 50). When hypersensitivity was well established (17 days for CCI, 21 days in STZ and 28 days in BTZ), a single bolus of PC1 (150 µg/kg, sc) was able to rapidly counteract the mechanical allodynia. In CCI mice, PC1 administration exerted an anti-allodynic effect in 30-120 min (46). In STZ mice the injection of PC1 produced a total recovery of PWT in 30 min, this anti-allodynic effect lasted for about 120 min and gradually disappeared within 240 min (48). In BTZ mice the effect of PC1 administration was maximal between 60 and 120 min and then progressively decreased, although it was still present after 240 min (50). In CIBP model, PKS was antagonized using neutralizing anti-Bv8 antibody (5 ng, IT) and a significant anti-hyperalgesic effect to mechanical stimuli was observed (45). In particular, this effect appeared at 15 min, peaked at 30 min and disappeared at 60 min after IT injection. Although in the different neuropathic models there are some differences in the rate and/or duration of the effect of acute PKS antagonism, it is possible to assert that the acute treatment with a PKS antagonist rapidly counteracts painful symptoms and its effect lasts for about



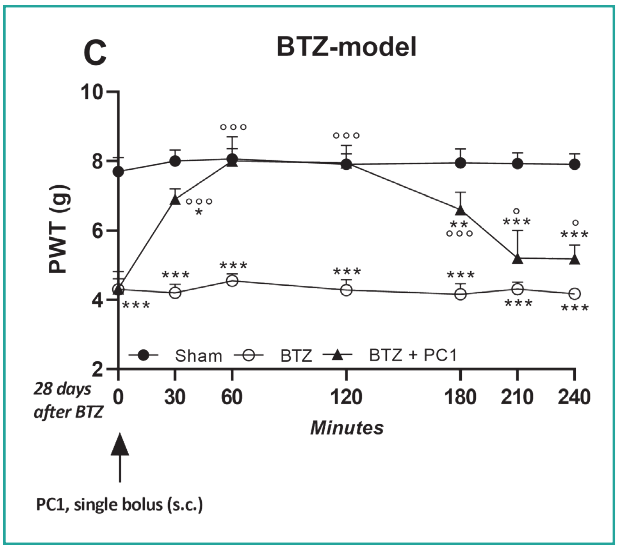


Figure 3. Acute effect of PC1 treatment in neuropathic pain models.
Acute effect on mechanical allodynia of single bolus of PC1 (150 µg/kg, s.c.) in CCI-mice at 17 days after injury (A, modified by Maftei *et al.*, (46)), STZ-mice at 21 days after injection (B, modified by Castelli et al., (48)) and BTZ-mice at 28 days after chemotherapic drug (C, modified by Moschetti *et al.*, (50)). PWT: Paw withdrawal threshold. Data present means ± SEM (A and B) or SD (C) of 5-6 mice per group. Statistical analysis has been performed by Two-Way ANOVA followed by Bonferroni's post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Sham; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ *vs* pathological group (CCI, STZ or BTZ).

4 hrs. Chronic treatment with PC1 (**figure 4**) has been performed either with a therapeutic approach, starting when pain was fully established (41, 46, 48-52) or in a preventive way, before the induction of the pathology (47, 48). In all the neuropathic models used, the chronic therapeutic treatment with PC1 was able to counteract painful symptoms, reducing allodynia and/or hyperalgesia. Interestingly, pain relief is maintained for few days also after PC1 treatment interruption (41, 46-48, 50). Considering that chemotherapy treatment is often repeated, and pain always reappears, it was also demonstrated that animals treated with PC1 during the first cycle of BTZ showed lower allodynia during the second chemotherapic cycle (50). In addition, Castelli and colleagues (48) also showed how preventive treatment with PC1 completely prevented the development of painful symptoms in diabetic mice. This preventive effect of PC1 treatment was observed also in SNI-animals (47), suggesting that a selective PKS antagonism could be an effective preventive approach.

PK2 and neuroinflammation

Neuroinflammation plays a key role in the onset and maintenance of several types of chron-

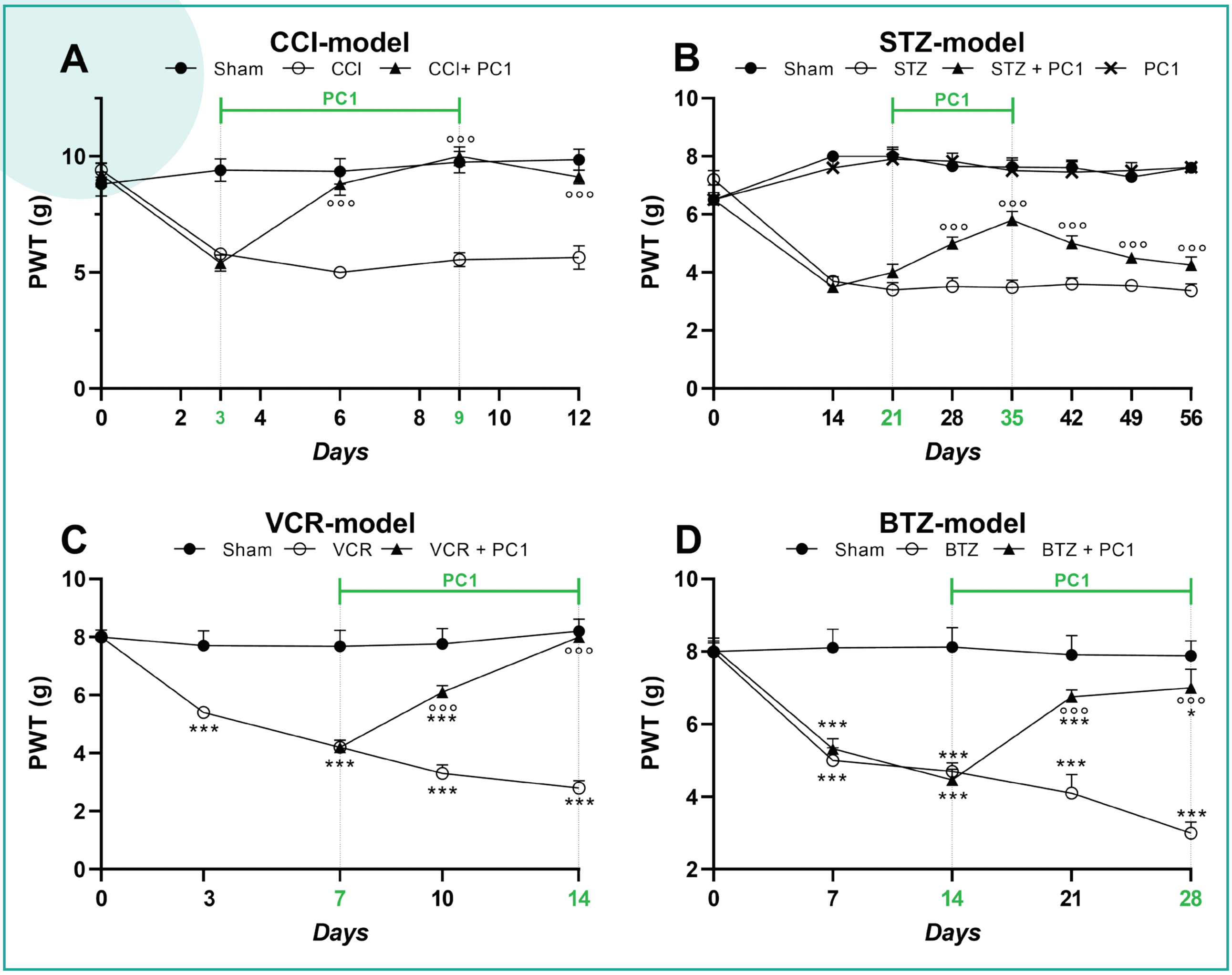


Figure 4. Effect of chronic PC1 treatment.
Therapeutic effect of chronic treatment by PC1 (150 μg/kg, s.c.) on mechanical allodynia in CCI-mice (A, modified by Lattanzi *et al*., (41)), STZ-mice (B, modified by Castelli *et al*., (48)), VCR-mice (C, modified by Moschetti *et al*., (49)) and BTZ-mice (D, modified by Moschetti *et al*., (50)). PWT: Paw withdrawal threshold. Data present means ± SEM (A-C) or SD (D) of 6-9 mice per group. Statistical analysis has been performed by Two-Way ANOVA followed by Bonferroni's post-test. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs* Sham; $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ *vs* pathological group (CCI, STZ, VCR or BTZ).

ic pain and has been clearly associated to neuropathic pain (53). Besides neurons, also glial and immune cells play an important role in this condition (54). Indeed, neuroinflammation is usually defined as a 'cytokine-mediated' inflammatory process (55). A peripheral damage to the nervous system induces the recruitment and activation of immune and glial cells in different anatomical sites (54). PK2 has a recognized pronociceptive and proinflammatory effect and is produced by immune cells, glial cells and neurons (41, 46-52, 56, 57). Its role in neuroinflammation has been studied in detail (**table I**). In all studies PKS members and other neuroinflammation markers were simultaneously evaluated in nervous stations involved in pain transmission and nociceptive processes, like sciatic nerve, dorsal root ganglia (DRGs), spinal cord and supraspinal areas.

Sciatic Nerve

In CCI animals, Lattanzi *et al.*, (41) found an early upregulation of PK2 expression in ipsilat-

Table I Prokineticin system and neuroinflammatory markers in neuropathic pain models.

	CCI model [41,46]	**SNI model** [47]	**STZ model** [48]	**VCR model** [49]	**BTZ model** [50,52]	**CIBP model** [45]
Sciatic Nerve	↑ PK2(*) ↑ PKR1 and PKR2	↑ PK2(*) ↑ PKR2	↑ PKR1(*)	-	↑ PK2(*)	
	↑ IL-1β(*), TNFα(*), IL-6(*) and IL-17(*)	-	↑ IL-1β(*)	-	↑ IL-1β(*), TNFα(*) and IL-6(*)	
	↓ IL-10(*)	-	↓ IL-10(*)	-	↓ IL-10	
	↑ CD11b ↑ GFAP(*) and S100β	-	-	-	↑ CD68(*) ↑ TLR4(*)	
Dorsal Root Ganglia	↑ PK2(*) ↑ PKR2	-	-	↑ PK2(*) ↑ PKR1(*) and PKR2(*)	↑ PK2(*) ↑ PKR1(*) and PKR2	
	-	-	-	↑ IL-1β(*), TNFα(*) and IL-6(*)	↑ IL-1β(*), TNFα(*) and IL-6(*)	
	-	-	-	↓ IL-10(*)	↓ IL-10	
	-	-	-	↑ CD68(*) and CD11b(*) ↑ TLR4(*)	↑ CD68(*) ↑ TLR4(*)	
Spinal Cord	↑ PK2(*) ↑ PKR2	↑ PK2(*) ↑ PKR2	↑ PK2 ↑ PKR2	↑ PK2(*) ↑ PKR1(*) and PKR2(*)	↑ PK2(*) ↑ PKR1(*) and PKR2(*)	↑ PK2
	↑ IL-1β(*)	-	↑ IL-1β(*)	↑ IL-1β(*) and TNFα(*)	↑ IL-1β(*) and IL-6	↑ TNFα(*)
	= IL-10	-	= IL-10	= IL-10	↓ IL-10(*)	
	↑ CD11b(*) ↑ GFAP(*)	↑ iba1(*) and CD206 ↑ GFAP(*)	-	↑ CD68(*) and CD11b(*) ↑ TLR4(*) ↑ GFAP	↑ CD68(*) ↑ TLR4(*) ↑ GFAP	
	-	-	-	-	↑ KDM6A(*)	

(*) PKS antagonism (by PC1 in CCI, SNI, STZ, VCR and BTZ mice; or by BV8 neutralizing antibody in CIBP rats) countered neuroinflammation induced by neuropathic pain.

eral sciatic nerves (3 days after CCI) and this overexpression was maintained up to 17 days after CCI. In accordance with these observations, Maftei *et al.* (46) observed in CCI-mice at day 10 post-surgery a strong infiltration of PK2+ cells in the proximity of the nerve damage. In this model also PKRs were upregulated in comparison to sham mice. PK2 and PKR2 levels were higher also in the other model of direct nerve damage, the SNI (47). In BTZ animals, PK2 levels were upregulated at 28 days after chemotherapy treatment, corresponding to a high cumulative dose, but not earlier (14 days) and in these mice PKRs mRNA levels were never modulated by the chemotherapeutic drug (50).

It is interesting to note that PKS modulation was always associated with an increase of neuroinflammatory markers (41, 46, 50). In detail, in CCI-mice (day 10) a significant up-regulation of pro-inflammatory cytokines (IL-1β, TNFα, IL-6 and IL-17) and down-regulation of IL-10 levels were detected (41, 46). Also in BTZ mice, neuroinflammation was present and was more sustained at day 28 (maximal cumulative dose, c.d.) than day 14 (half c.d.), indeed, at day 14 only CD68, TLR4 and IL-6 mRNA levels were increased, while at day 28 also TNFα and IL-1β were upregulated and IL-10 expression levels were reduced (50). Moreover, in both models (CCI and BTZ) an increase of activated macrophages (identified by CD11b+ cells in CCI and CD68+ cells in BTZ) was observed, and PK2 co-localized with these cells. Furthermore, in CCI-mice (41) a strong Schwann cells activation (GFAP+ and S100+ cells) was detected and PK2 co-localized with these cells.

In all neuropathic models the PKS antagonism with PC1 was able to restore correct PK2 levels (41, 46, 47, 50). Conversely, PC1 treatment did not modulate PKRs levels altered by pathology. Moreover, PC1 treatment was able to contrast or prevent neuroinflammation. Indeed, in STZ mice IL-1β levels were decreased and IL-10 levels were increased by PC1 therapeutic treatment (48). In CCI and BTZ mice, where the panel of neuroinflammatory markers was more extensive, it was possible to observe that PC1 treatment restored almost all of the parameters analyzed (41, 50). Interestingly, in CCI mice treated with PC1, the decrease of PK2 was associated with a decrease of GFAP+ cells (41, 46), while in BTZ mice treated with PC1 was associated with a decrease of CD68+ cells (50). It could be hypothesized that the block of PK2 by PC1 treatment, counteracts neuroinflammation, macrophage infiltration and Schwann cell activation.

Dorsal Root Ganglia

The PK2 time-course expression was also evaluated in DRGs of CCI, BTZ- and VCR- models (41, 49, 50). In this station PK2 expression levels appeared up-regulated from 7 to 17 days post-surgery in CCI, (41, 46) and also PKR2 was increased as both mRNA and protein. In both chemotherapy models, VCR and BTZ, PK2 levels resulted significantly up-regulated at the maximal c.d. (49, 50). Interestingly, a clear up-regulation of both PKRs resulted early in VCR mice (49), whereas in BTZ mice it was only present in the later stage (50). In addition, the levels of pro-/anti-inflammatory cytokines were also modified, albeit with different timings in the two models. In VCR mice, IL-1β up-regulation is precociously present, followed by increase of TNFα and IL-6 and decrease of IL-10 (49). Instead in BTZ mice, IL-6 and TNFα levels were increased and IL-10 levels decreased at the half c.d. while IL-1β levels were upregulated at the maximal c.d. (50). In BTZ and VCR model, a clear up-regulation of macrophage markers (CD68 and CD11b) and of TLR4 mRNA levels was detected, suggesting the presence of macrophage infiltration and activation (49, 50). In CCI mice, chronic PC1 treatment was able to restore disease-altered PK2 levels, but an effect on PKR2 was not detected (41, 46), whereas in chemotherapeutic treated mice it prevented PK2 up-regulation and contrasted PKR1 and/or PKR2 overexpression (49, 50). Moreover, the treatment with the antagonist of PKS restored or maintained at physiological levels all the inflammatory markers modified by chemotherapic treatment (49, 50). A detailed immunohistochemistry analysis revealed that in CCI mice, PK2 and PKRs were expressed by neurons with a vesicular cytoplasmic pattern which is dense in proximity of the neuronal membrane. In addition, PKS was also expressed by satellite cells since a clear colocalization of both PK2 and PKRs with GFAP+ cells was detected (41, 46). Moreover, in BTZ-mice the PK2 colocalized mainly with CD68+ cells (macrophages) (50). It is clear that in DRGs several cell types represent a source of PK2 in pain conditions.

Moschetti *et al.* (56) used primary cultures of DRG neurons to further investigate the role of PKS in chemotherapy induced neurotoxicity. The authors observed that VCR (1 nM) or BTZ

(6 nM) has a strong impact on neurons, significantly reducing neurite growth and length. This effect in VCR cultures was also associated with an increase in PK2, PKR1, TLR4, IL-1β, IL-6 and IL-10 mRNA levels. In co-culture with the chemotherapy drug (BTZ or VCR), PC1 prevented the reduction of neurite length, and the upregulation of the neuroinflammatory markers, protecting neurons from chemotherapy-induced toxicity. Interestingly, a protective role of PC1 for DRG cells was also observed *in vivo* (50). In BTZ mice swollen mitochondria and enlarged endoplasmic reticulum cisternae scattered within the cytoplasm of both nerve cell bodies and satellite glial cells were present (50). *In vivo* PC1 treatment was able to partially preserve neurons and satellite glial cell structure (50). Thus, in this station a neuropathic pain conditions of different ethiology induced the activation of PKS which participated in the onset or maintenance of pain. Besides, a clear neuroinflammation with pro- and anti-inflammatory cytokine unbalance, macrophage infiltration and satellite glial activation/alteration was detected. PKS antagonism was able to counteract or prevent it, suggesting the role of the system in these processes.

Spinal Cord

In the spinal cord, PK2 expression levels were evaluated in CCI, SNI, STZ, BTZ, VCR and CIBP models (41, 46-50, 52). In diabetic and CIBP models, when painful symptomatology appeared, an evident up-regulation of PK2 levels (mRNA and/or protein) was already present (46, 48). Moreover, PK2 levels were over-expressed as long as the mice were in pain. This suggests that PKS activation is involved in chronic pain development and chronicization. In STZ model, 35 days after toxin injection, PK2 increase was associated with PKR2 over-expression (48). Consistently with these data, also in CCI- (46) and SNI-models (47) at 10 days post-injury increased levels of PK2 and PKR2, were detected. A different activation of PKS was observed in chemotherapy models. In BTZ and VCR mice pain is already developed at lower dose but a significant increase of PK2 levels were detected only at the end of the experimental protocol, when the animals received the maximal c.d. (49, 50, 52). These data suggest that in chronic pain induced by chemotherapy treatment, central activation of PK2 is more associated with the maintenance rather than with the onset of pain. In spinal cord, PKS activation is always associated with a pronounced neuroinflammation. Indeed, in STZ, CCI, VCR and BTZ models a significant increase of IL-1β levels was always present (46, 48-50). Additionally, in all neuropathic pain models, it was detected an overexpression of glial markers, indicating the important role of this cellular component in pain. However, no colocalization between microglia cells and PK2 was ever observed. In SNI, CCI and BTZ models an increase of GFAP+ cells was described and it was demonstrated a co-localization of PK2 with both GFAP+ cells (astrocytes) and synaptophysin+ cells (neurons) (41, 46, 47, 50). This suggests that microglia and/or infiltrating macrophages do not represent the main source of PK2 in the spinal cord, which could therefore be produced by the astrocytic and neural components. The PKS antagonism was able to reduce PK2 overexpression in all neuropathic pain models. Down-regulation of PKRs was also detected, although the main effect was observed on PKR2 levels. Moreover, in all models a general reduction of neuroinflammation was present both for pro-inflammatory cytokines and glial cell activation markers. Finally, in a very recent paper, the possible interplay between PKS and epigenetic mechanisms in BTZ mice was proposed (52). The histone demethylase KDM6A, that has a role in promoting IL-6 production was up-regulated in mice with chronic pain. The antagonism of PKS with PC1 was able to prevent KDM6A alteration, controlling epigenetic mechanisms involved in cytokine production. Moreover, the paper also showed that by blocking PKS, the anti-inflammatory response sustained by PPARs was enhanced (52). In the whole these results suggest that also in the spinal cord PKS plays an important role in onset and/or maintenance of pain and neuroinflammation. PK2 produced by neurons and astrocytes induces the release of proinflammatory cytokines and epigenetic modifications that lead to microglia and astrocyte activation, triggering a proinflammatory loop that ends up with more PK2 production. The PKR antagonist interrupts this pathological loop that may be implicated in central sensitisation.

Supraspinal areas and mood alterations

In humans, the presence of chronic pain is frequently associated with mood alteration, such as depressive or anxious states (58). Also in experimental models of neuropathic pain, the development of anxious and/or depressive like behaviours has been reported (59). The effect of PKS antagonism on mood disorders in neuropathic mice was investigated in 2 papers (49, 51), which explored these aspects in chemotherapy-induced painful neuropathy. In BTZ-treated animals that had experienced chronic pain for 28 days, depressive and anxious behaviours were clearly present (51). The treatment with the PC1 antagonist, that as reported above, completely controlled painful symptoms, also counteracted mood alterations (51). Interestingly in VCR treated mice, who had been in a chronic pain condition only for 14 days, no mood alterations were recorded, suggesting that the duration of chronic pain may be important for the induction of neuropsychiatric alterations (49). In BTZ animals the presence of a neuroinflammatory condition in brain areas involved in anxiety and depression was also evaluated (51). A generalized neuroinflammation was observed with a significant mRNA level increase of CD11b in both prefrontal cortex and hypothalamus, of TRL4 in the prefrontal cortex and of GFAP in the hypothalamus. These results suggested the activation of both microglial and astrocytic components. Furthermore, the pro-inflammatory cytokines IL-6 and TNFα, that may be related to depressive condition, were up-regulated in prefrontal cortex, hippocampus and hypothalamus A drastic decrease in BDNF levels was also observed in the prefrontal cortex and hippocampus, condition widely correlated with depressive symptoms. Also PK2 was significantly increased in hypothalamus and hippocampus and an increment of PKR2 was observed in hippocampus. PKS antagonism with PC1 was able to prevent and/or counteract both neuroinflammation and BDNF decrease in these supraspinal areas. Consistently with the lack of mood alteration in VCR mice, no major alterations were observed in supraspinal areas in these animals (49).

CONCLUSIONS

From the evidence present in the literature we can affirm that PK2 overexpression is involved in the processes that underlie pain and neuroinflammation. An upregulation of this chemokine is consistently observed in nerves, DRG and spinal cords in models of peripheral neuropathic pain, independently of the causes (injury, diabetes, chemotherapic treatment) that induce pain. However, some differences in the time course of PK activation are present. For example, the system is immediately activated in models, such as CCI, where there is an immediate and strong local inflammatory response in the lesioned nerve with neurinoma formation, in comparison to chemotherapy induced neuropathy, where the PK system plays a delayed role that seems related to spinal sensitization. The results here summarized also demonstrate that both neurons and non-neuronal cells may express PK2. Besides neurons, infiltrating macrophages, DRG satellite cells and spinal astrocytes are important sources of the chemokine, that, on the contrary is not produced by spinal microglia. However, these cells express PK receptors and their activity is therefore modulated by prokineticins. The control of PK activation and of its effects with pharmacological antagonists, monoclonal antibodies or genetic strategies such as the generation of PK2 and PKR deficient animals, has proved to be a winning strategy to counteract pain and neuroinflammation. Interestingly an effective control of neuropathic pain with PK antagonists can also prevent the development of pain related comorbidity such as depressive and anxious-like behaviours.

Although other studies are needed to better dissect and understand the downstream pathways of PKS effects, we can affirm that PKS is an emerging excellent therapeutic target for the resolution of chronic pain and its comorbidities.

ETHICS

Conflict of interests

The authors declare that they have no conflict of interests.

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Availability of data and materials

Owned by third parties. The data underlying this article were provided by third parties, specified in the figure legends and cited in the references, under appropriate license or permission. Data may be shared after permission from the original authors.

Ethical approval

N/A

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