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CONTROLLING THE ACTIVATION OF THE PROKINETICIN SYSTEM REDUCES NEUROINFLAMMATION AND ABOLISHES PAIN HYPERSENSITIVITY IN EXPERIMENTAL NEUROPATHIC PAIN

Tesi di Dottorato di Ricerca

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TABLE OF CONTENTS

1. ABSTRACT	pag.05
2. INTRODUCTION	pag.10
2.1 ANATOMY AND PHYSIOLOGY OF PAIN	pag.11
2.1.1 PAIN PATHWAY	pag.12
2.1.2 PAIN MODULATION	pag.15
2.1.3 MECHANISMS INVOLVED IN THE TRANSITION FROM PHYSIOLOGICAL TO PATHOLOGICAL PAIN PROCESSING	pag.17
2.2 NEUROPATHIC PAIN	pag.19
2.2.1 NEUROPATHIC PAIN TRIAD (NEURONS, IMMUNE CELLS AND GLIA).....	pag.21
2.2.2 PHARMACOLOGICAL CONSIDERATIONS: PRESENT AND FUTURE	pag.25
2.3 DIABETES AND DEVELOPMENT OF NEUROPATHIC PAIN	pag.25
2.3.1 PAINFUL DIABETIC NEUROPATHY	pag.27
2.3.2 PATHOGENESIS OF DIABETIC NEUROPATHY	pag.28
2.4 MURINE MODELS OF NEUROPATHIC PAIN	pag.31
2.4.1 PERIPHERAL NERVE INJURY MODELS	pag.31
2.4.2 MODELS OF DIABETES-INDUCED PERIPHERAL NEUROPATHIC PAIN	pag.34
2.5 THE PROKINETICIN SYSTEM	pag.35
2.5.1 PROKINETICINS AND PROKINETICIN RECEPTORS: gene sequence, protein structure and anatomical localization	pag.36
2.5.2 BIOLOGICAL FUNCTIONS OF PROKINETICINS	pag.39
2.5.2.1 ROLE OF PROKINETICINS IN PAIN	pag.42
2.5.2.2 FUNCTION OF PROKINETICINS IN IMMUNE SYSTEM	pag.43
2.5.3 PROKINETICIN RECEPTOR ANTAGONISTS	pag.45
3. AIM OF THE WORK	pag.47
4. MATERIALS and METHODS	pag.50
4.1 ANIMALS	pag.51
4.2 INDUCTION OF PAINFUL NEUROPATHY	pag.51
4.2.1 PERIPHERAL NERVE INJURY MODEL	pag.51
4.2.2 DIABETES-INDUCED NEUROPATHIC PAIN MODEL	pag.51
4.3 EXPERIMENTAL DESIGN	pag.52
4.3.1 TREATMENT	pag.52
4.3.2 CHARACTERIZATION OF NOCICEPTIVE BEHAVIOUR	pag.54
4.3.3 TISSUE AND CELL SAMPLING	pag.54
4.4 NOCICEPTIVE BEHAVIOURAL TESTS	pag.57
4.4.1 PLANTAR TEST: THERMAL HYPERALGESIA EVALUATION	pag.57
4.4.2 VON FREY TEST: MECHANICAL ALLODYNIA EVALUATION	pag.58
4.5 BIOCHEMICAL EVALUATION	pag.59
4.5.1 REAL TIME PCR	pag.59
4.5.2 ENZYME-LINKED IMMUNO-SORBENT ASSAY (ELISA)	pag.60

4.5.3 WESTERN BLOT	pag.61
4.6 HISTOCHEMICAL EVALUATION	pag.62
4.6.1 IMMUNOFLUORESCENCE	pag.62
4.6.2 SCIATIC NERVE IMMUNOHISTOCHEMISTRY	pag.63
4.7 DATA ANALYSIS	pag.63
5. RESULTS	pag.64
5.1 PERIPHERAL NERVE INJURY (CCI) MODEL	pag.65
5.1.1 Effect of PKR blocking on CCI-induced thermal hyperalgesia and mechanical allodynia	pag.65
5.1.2 Prokineticin system localization and CCI-induced modulation	pag.67
5.1.3 Effect of therapeutic PKR blocking on cytokines levels in spinal cord and sciatic nerve	pag.72
5.2 DIABETES-INDUCED NEUROPATHIC PAIN MODEL	pag.73
5.2.1 Effect of therapeutic PKR blocking on hyperglycaemia, mechanical allodynia and body weight loss in experimental diabetic model induced by the administration of a single high dose or multi-lower doses of STZ: similarities and differences	pag.73
5.2.2 Prokineticin system and diabetic neuropathic pain-modulation	pag.76
5.2.3 Effect of therapeutic PKR blocking on cytokines levels in spinal cord and sciatic nerve	pag.81
5.2.4 Effect of PKR blocking on peripheral inflammatory status and immune dysfunction associated to the development of diabetes	pag.83
6. DISCUSSION and CONCLUSIONS	pag.89
7. REFERENCES	pag.97
8. LIST OF PUBLICATIONS	pag.111

1. ABSTRACT

Neuropathic pain is a chronic and debilitating disease that occurs secondarily to injury of the peripheral and/or central nervous system. This pathology affects million people in the world and can be classified as an incurable disease for the lack of valid treatments.

Neuronal injuries often arise from a nerve trauma or metabolic disease, such as diabetes, and neuropathic patients, whatever the cause, typically exhibit a mixture of sensory loss with ongoing spontaneous pain and enhanced sensitivity either to innocuous or painful stimuli. Although the underlying mechanisms are far to being elucidated, it is well established that neuronal injury not only results in profound modifications in the activity of sensory neurons and their central projection pathways, but is also coupled to a sustained immune response at different anatomical locations associated to chronic pain processing with an important contribution of cytokines and chemokines (Calvo et al., 2012; Sacerdote et al., 2013).

Since intensive researches over the past years have identified the prokineticins (PKs) as possible candidates for mediating these pathological neuro-immune interactions in pain, in these years of PhD school my research was focused on the characterization of the PKs system in the development of experimental neuropathic pain.

PKs family comprehends small chemokines-like proteins highly conserved across the species including the mammalian prokineticin 1 (PK1) and prokineticin 2 (PK2). These proteins modulate a large spectrum of biological activities in the organism. In particular it is well documented the pro-nociceptive/proinflammatory activity of the ligand PK2 (Negri et al., 2007). Two G protein-coupled receptors (PKR1 and PKR2) mediate PK2 actions.

PK2, binding to PKR1 and PKR2 widely distributed in the central nervous system, DRG, sensory neurons and in cells participating to immune and inflammatory responses, exerts in fact a critical role in pain perception inducing nociceptor sensitization and increasing the release of neuromediators implicated in pain processing such as CGRP and SP (Negri et al., 2007; DeFelice et al., 2012; Vellani et al., 2006). Moreover the ligand influences macrophages and T lymphocytes activity inducing a pro-inflammatory phenotype in the macrophage and skewing the Th1/Th2 balance towards a Th1 response (Martucci et al., 2006; Franchi et al., 2008).

In order to understand if PK2, PKR1 and PKR2 activities were necessary for the onset, maintenance and resolution of neuropathic pain, in this study, in vivo and ex-vivo experiments were performed using a non-peptidic PKR antagonist, named PC1, proved capable of antagonizing all pro-nociceptive effects induced by PK2 (Balboni et al., 2008; Giannini et al., 2009; Negri and Lattanzi, 2012).

The efficacy of PC1 treatment was evaluated in two different mouse models of painful neuropathy: a mononeuropathy induced by the chronic constriction injury (CCI) of sciatic nerve and a diabetic polyneuropathy induced by the injection of a pancreatic β cell toxin, streptozotocin (STZ).

CCI procedure was performed through three loose ligatures around the right common sciatic nerve while the diabetic painful neuropathy was induced in animals by the administration of either a single high dose (200 mg/kg) or repeated multi-lower doses (80 mg/kg) of STZ. Changes in pain behavior were evaluated measuring the paw withdrawal thresholds after noxious (hyperalgesia) and/or innocuous (allodynia) stimulation with the Plantar Test Apparatus and the Dynamic Plantar Aesthesiometer, respectively.

To check the efficacy of PC1 to counteract painful manifestations, 3 days after CCI surgery and 21 days after STZ administrations, time points corresponding to full neuropathic pain development, CCI-operated and STZ-injected mice were subjected to a therapeutic treatment with the antagonist PC1 (150 µg/kg).

The first major finding of this study was that, independently from neuropathic pain etiology, PC1 treatment was effective in alleviating established painful symptoms in mice without producing tolerance. Repeated systemic injections of PC1 from day 3 to 9 after surgery or from day 21 to 34 after diabetes induction in fact abolished thermal hyperalgesia and mechanical allodynia in nerve injured mice, and mechanical allodynia in diabetic animals.

The fact that painful symptoms were completely reversed by the chronic administration of the PKR antagonist unequivocally indicated the involvement of the PKs system in neuropathic pain. Moreover, interestingly, in STZ-injected mice the anti-allodynic effect induced by the antagonist was still evident two weeks after the treatment discontinuation leading us to suppose that blocking PK2 signaling could induce permanent changes in neuronal circuits involved in the maintenance of neuropathic pain.

At the end of treatments, i.e. on day 10 after CCI surgery and at different time points from diabetes induction (7, 14, 35 and 56 days after STZ injection) when the anti-hyperalgesic and anti-allodynic effects of PC1 were evident, biochemical evaluations were performed in neuropathic animals (CCI-operated and STZ-injected mice) treated with either PC1 or saline and in the respective controls to determine the expression of PK2 and its receptors, PKR1 and PKR2, at the peripheral and central sites of pain transmission.

Real Time PCR analysis performed on sciatic nerve and spinal cord from neuropathic animals revealed a general up-regulation of PK2 and PKRs in these tissues furthermore demonstrating the close correlation between the PKs system and the development of neuropathic pain. In particular, in STZ model, an over expression of PK2 in spinal cord was present since the appearance of painful symptoms and was observed for all the persistence of allodynia.

In addition, we also exactly discriminated in the spinal cord and in periphery, the cells mainly involved in the CCI-induced PKs system activation. In the spinal cord of injured nerve mice the expression of PK2 and PKRs was observed in the superficial layers of

the spinal cord, at the levels of the presynaptic terminals. PK2 as well as PKR2 were also mostly expressed in proliferating and activated astrocytes. In periphery, at the level of the injured nerve, the expression of PK2 was evident in Schwann cells, neutrophils and macrophages, while PKR1 and PKR2 were highly expressed on activated inflammatory cells and on Schwann cells, respectively.

In CCI animals the therapeutic treatment with the antagonist PC1 succeeded in decreasing the neuropathy-induced PK2 up-regulation both in the spinal cord and in the injured nerve, without significantly affecting PKR1 and PKR2 mRNA levels. In particular, a significant reduction of PK2 immunoreactivity was observed at the presynaptic terminals of the dorsal horns, in the reactive spinal astrocytes and in infiltrating neutrophils, mirroring the lower PK2 mRNA levels.

In STZ mice, the therapeutic treatment with the antagonist was also able to counteract the PK2 augmentation in the spinal cord and to significantly reduce the neuropathy-induced PKR1 up-regulation in the sciatic nerve. Since PKR1 is the receptor mostly implicated in the immune response and it was previously demonstrated to mediate macrophage migration (Martucci et al., 2006), it can be assumed that blocking PKRs with PC1 could affect macrophage chemotaxis, reducing or preventing the recruitment of inflammatory cells expressing PKR1 in the nerve with a consequence reduction of neuroinflammation.

Considering the pro-inflammatory activity of PK2 and the presence of the PKRs in Schwann and immune cells in the nerve and the PKR2 in the spinal astrocytes, it was examined the efficacy of PC1 to counteract also the neuroinflammation associated to neuropathic pain development, evaluating by Real Time PCR and ELISA, the levels of the pro-inflammatory cytokine IL-1 β and anti-inflammatory cytokine IL-10 in the sciatic nerve and the spinal cord from neuropathic mice. The release of inflammatory mediators, such as cytokines and chemokines, from glia and immune cells plays in fact an important role in the genesis of neuropathic pain and it was demonstrated that an altered balance of some pro- and anti-inflammatory cytokines in nervous tissues linked to pain transmission, such as the nerve, the DRG and the spinal cord is well correlated with the presence of neuropathic pain either in CCI or STZ mice (Sacerdote et al., 2013; Valsecchi et al., 2011).

In agreement with what already published, in presence of high levels of PK2 and consistently with its immunomodulatory activity, an augmentation of the pro-nociceptive cytokine IL-1 β was observed both in the central and peripheral nervous system of CCI and STZ neuropathic mice, while the levels of the anti-inflammatory cytokine IL-10 appeared lower respect to the basal levels of controls.

Repeated PC1 administration induced a clear reduction of the neuropathy-induced IL-1 β increase observed in the sciatic nerve and in the spinal cord from neuropathic mice. In addition, PC1 enhanced the levels of IL-10, which is likely to participate in the therapeutic effects observed.

These data clearly demonstrated the implication of the PKs system in neuropathic pain suggesting its possible implication not only in the maintenance but also in the onset of the pathology. In order to confirm this hypothesis, we performed a precocious blocking of the PKRs in STZ mice not yet neuropathic. Early PC1 administrations from day 0, time point corresponding to first STZ injection, to 13 days after diabetes induction, prevented in fact the development of mechanical allodynia in STZ mice and the spinal cord up-regulation of PK2.

Glutamate is one of the main mediator in pain processing and it is known to participate in the alteration of the synaptic transmission during neuropathic pain (Iwata et al., 2007; Daulhac et., 2011). In order to further support the anti-allodynic effect of PC1, we analyzed the expression of glutamate NMDA and AMPA receptor subunits in spinal cord of STZ mice treated with preventive PC1 administrations.

Western blot analysis revealed that in presence of a fully developed allodynia, a decrease of the spinal NMDA subunit N2A was present, while the expression of the subunit N2B significantly increased. Early PC1 administration was effective in preventing N2B up-regulation in spinal cord of diabetic mice, without affecting the levels of the subunit N2A.

Finally, considering the precocious involvement of the PKs system in the onset of the diabetic neuropathy it was interesting to investigate whether a preventive blocking of the PKRs positively influenced also the course of the diabetic pathology itself, modulating the hyperglycaemic state of the animals or reducing the peripheral inflammatory component which is known to be associated to diabetic status (Agrawal and Kant, 2014).

Early PC1 administrations from day 0 to 13 after diabetes induction were not effective either in reducing high glucose levels in STZ mice or in re-establishing the plasmatic insulin levels. However, blocking the PKs system was effective in ameliorating the general pro-inflammatory status that was present in diabetic mice. The antagonist was in fact able to prevent the dysregulation of the IL-1 β and IL-10 levels in the pancreas, which appeared drastically diminished in the STZ mice.

Moreover, in the diabetic animals we observed a significant alteration of both innate and acquired immunity, characterized by elevated levels of IL-1 β produced by macrophages, and a Th1 pro-inflammatory profile. The PC1 treatment reduced the peripheral inflammatory status, decreasing macrophagic IL-1 β and switching Th1/Th2 balance towards Th2.

In conclusion, considering the efficacy of PC1 to contrast painful symptoms and the neuroinflammation associated to the development of neuropathic pain, blocking PKRs signalling could represent a new possible therapeutic strategy to treat neuropathic pain. In addition, beyond reducing the neuropathy-induced pain hypersensitivity, the anti-inflammatory properties of the antagonist PC1 could be useful to ameliorate other pathologies, characterized by a sustained inflammatory component.

2. INTRODUCTION

2.1 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 takes into consideration two important concepts concerning 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 between 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).

Pain can last for a short (acute pain) or long (chronic pain) 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 needful for maintaining body integrity as it represents a primitive defence mechanism evolved for warning us about the presence of potential dangerous stimuli and for minimizing 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; Raouf et al., 2010).

Also the inflammatory pain is physiological and protective. This kind of pain takes place in presence of tissue injuries or infections and is associated to an inflammatory state caused by the activation of the immune system. However the increased pain hypersensitivity is functional because protects the injured tissue site until healing occurs, by minimizing contact with stimuli potentially able to provoke other pain.

Usually, either nociceptive or inflammatory pain trigger adaptive responses and 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 maladaptive, not protective 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 rather a disease of the nervous system (Woolf, 2010).

2.1.1 PAIN PATHWAY

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

- I. Transduction of noxious stimuli (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 end 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 forks into 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, once coming 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 supraspinal structures and, finally the cortical and subcortical regions, where painful information is interpreted and perceived.

Unlike to other classes of sensory fibres, nociceptors have high activation thresholds; in fact in normal conditions, they can be excited only by stimuli enough intense to cause a real tissue damage, but not by innocuous stimulations such as light touch,

vibrations or warning. High threshold stimuli for nociceptors activation include extreme temperatures, intense pressure and irritant chemicals (Dubin and Patapoutian, 2010).

The main primary afferent fibres 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 A δ fibres and the unmyelinated, polymodal, i.e. able to detect mechanical, thermal and chemical stimuli, C fibres (Julius and Basbaum, 2001).

All A δ fibres response to intense mechanical stimulations but depending on their responsiveness to noxious heat can be divided in type I and type II fibres. The first type includes capsaicin-insensitive fibres responding to high temperatures (52-56 °C), while the type II population comprehends capsaicin-sensitive fibres responsive to noxious heat of 40-45 °C (Giordano, 2005).

Unmyelinated C fibres represent the majority of the primary afferents fibres and, according to ability of synthesizing neuropeptides, mostly the substance P (SP) and the calcitonin gene related peptide (CGRP), are broadly divided in two main populations, the peptidergic and the non-peptidergic.

A δ and C nociceptive fibres differ also in terms of conduction velocity. When activated, A δ fibres transmit nociceptive potentials with a velocity ranging to 12-30 m/s leading to a rapid pain sensation, while C fibres 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 successive and qualitatively distinct pain sensations: 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 fibres 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) A β fibres arising from DRG large-diameter neurons, responsive to mechanical innocuous stimuli. Normally they don't contribute to pain sensation but in some cases they begin to signal pain in response to non-noxious stimuli. Information transmit by A β fibres can be in fact greatly altered during disease conditions or after tissue damage has resolved, leading to abnormal pain signalling (Milligan and Watkins, 2009).

The information collected in periphery by nociceptive fibres reach 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 A δ and C fibres, Wide Dynamic Range (WDR) neurons that respond to

both nociceptive and non-nociceptive stimuli coming from A δ , C and A β central axons and NonNociceptive (N-NOC) neurons responding to innocuous stimulations mostly propagated by A β fibres (Almeida et al., 2004).

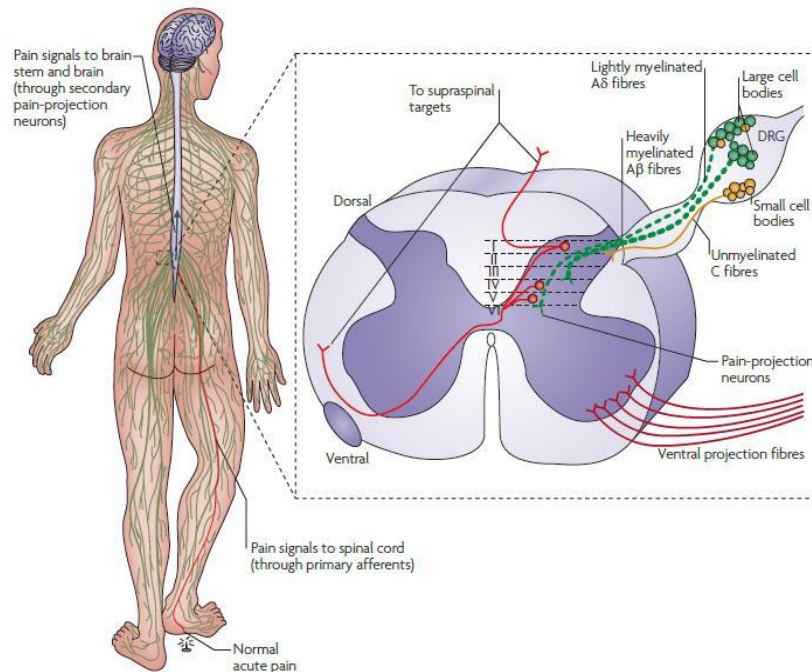


Figure 1 | **Nociception.** Figure taken from Milligan and Watkins, 2009.

Nociceptive fibres transmit the nociceptive message through the release of glutamate, as the primary neurotransmitter, and other 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 (Tracey and Johns, 2010; 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 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 tract 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 mechanisms responsible for the inhibition of the afference of this same pathway (Almeida et al., 2004).

2.1.2 PAIN MODULATION

The sensory experience of pain extends beyond the activation of nociceptors as it involves an intricate neuronal network consisting of dynamic excitatory and inhibitory nervous circuits which modulate pain experience 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's in the Gate Control Theory of Pain, 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. A β non-nociceptive fiber activity inhibits (or "closes") the gate inducing the activation of local inhibitory interneurons, while the activity of small nociceptive C and A δ fibres facilitates (or "opens") the gate (Moayedi and Davis, 2013). The balance between the 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 strong 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).

Pain modulation exists in fact also in the form of descending inhibitory and facilitatory pain pathways 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 that send projections to the spinal cord. These modulatory effects are predominantly mediated by descending pathways that utilize serotonin, norepinephrine and endogenous opioids.

Monoamines and endogenous opioids modulate the release of neurotransmitters from nociceptive afferents and the excitability of dorsal horn neurons by binding to 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 of pain transmission which is dependent on the receptor subtype activated (Suzuki et al., 2004; Dogrul et al., 2009).

Norepinephrine also strongly contributes in anti-nociceptive mechanisms associated to descending inhibition. PAG and RVM communicate with an important noradrenergic site to pain modulation, 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 (Field et al., 1991; 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. (Fields and Basbaum, 1999; Mason, 1999; Fields, 2000).

2.1.3 MECHANISMS 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 phenomena 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 capacity 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 primary afferents terminals and 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 (Campana, 2007).

“Inflammatory soup” includes prostaglandins E₂ (PGE₂), bradikinin, ATP, protons, nerve growth factor (NGF) and pro-inflammatory cytokines (TNF- α and IL-1 β) (Julius and Basbaum, 2001).

These mediators stimulate/activate nociceptors directly (e.g. protons, ATP and 5-hydroxytryptamine (5-HT)) or by increasing sensitivity to subsequent stimuli (e.g. bradikinin, 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, the 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 signalling 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-1 β . 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 others neuromodulators. Under normal, non-pathological condition, low-frequency activation of A δ and C fibre 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 postsynaptic 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 Mg²⁺ (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 et al., 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 Mg²⁺-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 A β 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).

2.2 NEUROPATHIC PAIN

Neuropathic pain is a pathological pain that occurs secondarily to injury of the central and/or peripheral nervous system. The *International Association for the Study of Pain (IASP)* has recently modified its definition as “pain caused by a lesion or disease of the somatosensory system” to emphasized that the injury in the nervous system has to be within the somatosensory system (Treede et al., 2008).

Neuropathic pain is an emerging pathology affecting million people in the world and can be classified as an incurable disease for the lack of valid treatments. In fact it is partially unresponsive to classical analgesics and its treatment with adjuvant drugs, i.e. antidepressant or anticonvulsive, provides only a temporary relief of pain in a small percentage of patients.

This chronic pain is highly invalidating because it directly impacts on lifestyle of sufferers who very often are forced to leave their work influencing the social life and psychological and emotional condition characteristic of the individual. This obviously poses enormous costs to society in terms of healthcare and social care .

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.

Whatever the cause, neuropathic patients typically exhibit a mixture of sensory loss with ongoing spontaneous pain and enhanced sensitivity to innocuous stimuli, i.e. mechanical allodynia. Mechanical and thermal hyperalgesia (increased pain response to painful stimuli) are also frequent and classical symptoms (Treede et al., 2008; Zimmermann, 2001; Colleoni and Sacerdote, 2010).

Much 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 (Teschke 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. nerve, DRG, spinal cord and brain (Calvo et al., 2012).

2.2.1 NEUROPATHIC PAIN TRIAD (NEURONS, IMMUNE CELLS AND GLIA)

Peripheral nerve injury provokes the recruitment of immune cells as well as the activation of resident cells at the site of injured nerve, in the DRG, and in the spinal cord. Communication among immune cells and immune-like glial cells along the way 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 state.

- 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 chemoattractants 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, forms at the lesion site (Scholz and Woolf, 2007).

Resident and infiltrating immune cells as well as Schwann cells release pro-inflammatory cytokines, such as TNF- α , IL-1 β , 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 WLDs 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.

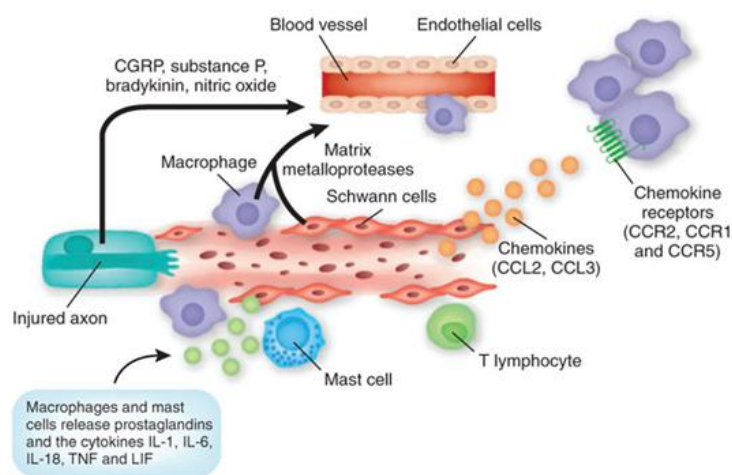


Figure 2 | **Inflammatory changes associated to Wallerian degeneration.**
Figure taken from Scholz and Woolf, 2007.

- Neuroimmune interactions in DRG

Normally, macrophages and few T lymphocytes reside in DRG. Their numbers increase after nerve damage. In parallel satellite glia cells begin to proliferate.

These resident immune and glia cells strongly react to nerve injury and their response is reinforced by invading macrophages and T cells. Injury-induced macrophage 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-1 β , 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

phenotype of sensory neurons 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-1 β receptor or IL-1RA overexpression inhibit the development of spontaneous sensory neuron firing, while blocking IL-1 β 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.

The release of inflammatory mediators, such as TNF- α , IL-1 β , 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-1 β 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-1 β receptor and presynaptic glutamate receptors (NMDAR) (Grace et al., 2014).

TNF- α and IL-1 β 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 glutamate AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, which render neurons vulnerable to excitotoxicity (Beattie et al., 2002; Stellwagen and Malenka, 2006).

IL-1 β also induces the phosphorylation of specific NMDA receptor subunits (i.e. NR1 and NR2A or NR2B subunits) leading to its activation (Stellwagen et al., 2005; 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-1 β 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 highlight 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).

2.2.2 PHARMACOLOGICAL CONSIDERATIONS: PRESENT AND FUTURE

Pain processing involves multi-pathways and dynamic systems in the periphery, spinal cord and brain which expose potential pharmacological targets for analgesia. Some of these are already used in clinic by existing analgesics (Farquhar-Smith, 2008).

Opioids are among the most powerful of all 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 transmission and pain perception.

The fact that most of pain inhibitory pathways are noradrenaline/serotonin-based pathways indicate why the antidepressants (inhibitor of noradrenaline/serotonine re-uptake) may be effective in controlling pain.

Anticonvulsants induce analgesia acting on neuron excitability through the blocking of sodium and calcium channels; similarly capsaicin exerts its analgesic effect binding to TRPV1 channel. Non-steroidal anti-inflammatory drugs (NSAID), 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 functions for pain management. Several promising strategies to target the neuroimmune interface include the direct inhibition of pro-inflammatory signalling, the 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.

2.3 DIABETES AND DEVELOPMENT OF NEUROPATHIC PAIN

Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic hyperglycaemia with impaired metabolism of carbohydrate, fat and proteins as a result of endogenous insulin deficiency and/or resistance (Davey et al., 2014).

Polydipsia, polyphagia, polyuria, blindness, weight loss or gain, burning and tingling sensation are some of distinct symptoms associated to diabetic status and are

common for the two main forms of diabetes: type 1 (DMT1) and type 2 (DMT2) diabetes.

Both types of diabetes are characterized by a progressive failure of pancreatic β -cells but the mechanisms leading to pancreatic cell death are quite different in the various forms of the disease (Cnop et al., 2005). In type 1 diabetes β -cell destruction arises from an autoimmune assault against pancreatic cells by autoreactive T lymphocytes resulting in chronic pancreatic inflammation (processes known as insulinitis) which culminates with an absolute insulin deficiency. During insulinitis, invading immune cells, including Th1 (CD4 and CD8) lymphocytes, macrophages and dendritic cells, participate to destruction of pancreatic β -cells by directly triggering cytotoxic processes or releasing pro-inflammatory cytokines.

IL-1 β , IFN- γ and TNF- α are important pro-inflammatory mediators. These cytokines induce β -cell apoptosis via the activation of pancreatic cell gene networks under the control of different transcription factors, including Nf-kB. The activation of Nf-kB leads to nitric oxide and chemokines production and depletion of endoplasmic reticulum calcium, which ultimately contribute to pancreatic cell destruction. Parallel, anti-inflammatory cytokines, such as IL-4 and IL-10, produced by activated Th2 lymphocytes, prevent β -cell destructive insulinitis, indicating that an imbalance between pro- and anti-inflammatory cytokines could be essential for the development of type 1 diabetes (Amirshahroki and Ghazi-Khansari, 2012).

The pathogenesis of type 2 diabetes is more variable than that of type 1 diabetes as it is linked to a combination of genetic and lifestyle factors that results in different degrees of insulin resistance and deficiency. Chronic exposure to elevated glucose and free fatty acids causes β -cell dysfunction and may induce β -cell apoptosis in type 2 diabetes (Cnop et al., 2005). Islet cell inflammation as result of altered immune activation has since long time been recognized in type 1 diabetes, and now it is increasingly implicated in the pathogenesis of type 2 diabetes leading to defects in β -cell secretion (Das and Mukhopadhyay, 2011).

Inflammation and alteration in immune system thus result common underpinning mechanisms in the pathophysiology of type 1 and type 2 diabetes as well as of their complications (Agrawal and Kant., 2014).

Macrophage, in particular, is the major source of pro-inflammatory cytokines and emerges as a key player in the initiation and maintenance of chronic inflammatory responses in diabetes by programming through the release of IL-12 cytokine T lymphocytes to develop into Th1 cells (Wen et al., 2006). Elevation of systemic inflammatory mediators was found in patients with type 1 and type 2 diabetes (Agrawal and Kant, 2014; Davey et al., 2014).

The establishment of a persistent hyperglycaemic state in diabetes causes a series of physiological dysfunctions in the organism which, over time, turn into very serious

complications responsible of the high rate of morbidity and mortality in diabetes sufferers.

These complications are associated to a progressive, dramatic failure and dysfunction of the vascular system. They are grouped in “macro-vascular complications” and “micro-vascular complications” depending on the vascular district which is affected.

Macro-vascular complications result from damage to arteries that supply the heart, brain, and lower extremities and include accelerated cardiovascular disease, peripheral arterial disease, myocardial infarction, stroke and limb amputation (Hofmann and Brownlee, 2004; Forbes and Cooper, 2013).

The complications resulting from damage to small blood vessels are grouped as microvascular complications. Persistent chronic hyperglycemia resulting in the development of diabetes-specific microvascular complications in the retina, renal glomerulus, and peripheral nerves are characteristic of all forms of diabetes. Microvascular complications are classified into retinopathy, nephropathy, and neuropathy (Davey et al., 2014).

2.3.1 PAINFUL DIABETIC NEUROPATHY

Diabetic neuropathy (DN) is one of the most frequent long-term complications of diabetes. It affects about 60% of diabetic population and is a source of morbidity and mortality in diabetic patients (Aslam et al., 2014). This type of peripheral nervous disorder is characterized by a progressive neuronal death, demyelination and suppression of the nerve regeneration mechanisms, resulting in an impaired nerve functioning.

Nerve damage can involve both the autonomic and the sensorimotor divisions of the nervous system, so every nerve fibre in the body is potentially vulnerable.

Diabetic neuropathic patients typically experience lack of sensibility in areas of the body and lose the capacity to judge the temperature or sense even painful, with a consequent impairment of the quality of life. Further, the progressive loss of innervations can lead to atrophy of essential pedal muscles, resulting in deformities that predispose the patient to ulceration and in the more severe cases to lower extremities amputation (Duby et al., 2004). Severity of symptoms increases gradually over time and correlate with the degree of hyperglycaemia (Han et al., 2013).

There are many types of DN. Depending on the organ systems and the types of nerves affected and the entity of the nerve damage, which can be diffused or well localized, diabetic neuropathies are classified in diffuse somatic neuropathy, autonomic neuropathy and focal neuropathy. Among these, the sensorimotor neuropathy or distal symmetrical polyneuropathy (DSPN) is the most common form.

DSPN affects both large and small sensory fibres resulting in a mixture of symptoms and sensory loss. The onset of the neuropathy is usually gradual and insidious and is

heralded by sensory symptoms that start from the most distal extremities of the limbs (toes and fingers) and progress proximally in a symmetrical “glove and stocking” distribution (Tesfaye et al., 2013). The syndrome involves initially the lower limbs.

Approximately 50% of patients with DSPN exhibits neuropathic pain symptoms, which include uncomfortable tingling in the lower limbs (dysesthesia), spontaneous and evoked pain and numbness. Unusual sensations such as feeling of swelling of the feet or severe coldness of the legs when clearly the lower limbs look and feel fine, odd sensations on walking likened to “walking on pebbles” or “walking on hot sand” are other characteristic clinical manifestations of the disease (Tesfaye et al., 2013). The pain is often worse at night and interferes with normal sleep causing tiredness during the day. The constant unremitting pain negatively impacts on the quality of life of diabetic patients resulting in form of depressions; in extreme cases patients experience loss of appetite and body weight known as “diabetic neuropathic cachexia” (Aslam et al., 2014).

The exact pathophysiology of diabetic neuropathic pain is not fully understood. However, the abnormalities of pain processing in the peripheral and central nervous system which are supposed to contribute to the development and maintenance of neuropathic pain (see chapter 2.2) could be related to hyperglycaemia, as this is the key metabolic abnormality of diabetes (Aslam et al., 2014).

2.3.2 PATHOGENESIS OF DIABETIC NEUROPATHY

Despite decades of intensive researches, the pathogenesis of the diabetic neuropathy has not been yet fully elucidated. Hyperglycaemia is considered to be one of the major pathophysiological determinants of the disease. However, the cause of this syndrome is more complex than dysregulated glucose levels alone (Han et al., 2013). Several factors have been postulated to participate in the DN pathogenesis, including microcirculatory dysfunction, impaired insulin signalling, growth factor deficiency and inflammation.

All these abnormalities are intertwined through numerous competing or parallel pathways and are supposed to contribute to a pathological self-perpetuating cycle of oxidative stress, inflammation and cellular dysfunction ultimately resulting in the progression of neurovascular disease associated to loss of nerve fibres.

Polyol pathway, advanced glycation end-product (AGE) production, poly-ADP ribose polymerase (PARP) over activation, protein kinase C (PKC) and altered Na^+/K^+ ATPase activity are some of the molecular mechanisms underlying DN. (Sandireddy et al., 2014).

- HYPERGLYCAEMIA

Prolonged hyperglycaemic state contributes to cell damage and, thus to neuropathy, through the generation of oxidative stress, as the final result of increased glycolysis, polyol pathway activity and generation of AGE products. Excess of glycolysis overloads the mitochondrial electron transport chain promoting the production of reactive oxygen species (ROS), while the abnormal high rate glucose “flux” through the polyol pathway leads to NADPH depletion causing oxidative stress.

Accumulation of reactive oxygen species (ROS) increases lipid, DNA and protein peroxidation, induces cellular apoptosis, reduces nerve blood flow (NBF) and induces impairment of vasodilation of epineural blood vessels, which results in ischemia to the neural tissue. Oxidative stress also leads to deterioration of Schwann cells, which play a key role as a provider of insulation for neurons, immunologic perineurial blood nerve barrier, and effector of nerve regeneration (Han et al., 2013).

The production of AGEs can impair cellular function by altering the structure and so the biological function of essential proteins. Moreover, binding to receptors (RAGE), they can trigger an inflammatory cascade that involves the activation of MAPK and PKC pathways and that ultimately generate oxidative stress (Duby et al., 2004).

- IMPAIRED INSULIN SIGNALLING

Insulin is essential for general neuronal function as it promotes neuronal growth and survival. Insulin receptors are present in DRG sensory neurons and in peripheral axons sustaining epidermis and a their increase was found after physical injury of peripheral nerves and in diabetes (Guo et al., 2011).

In diabetic mice, local or intranasal insulin administrations improve sensory nerve fibre density in the plantar foot pad and mechanical sensation (Francis et al., 2009).

In DMT1 patients, the reduction of C-peptide, marker for pancreatic β -cells functionality, contributes to nerve dysfunction by reducing the activity of Na^+/K^+ ATPase and eNOS and the endoneurial blood flow.

Treatment with C-peptide can slow down the progression of neuropathy (Ekberg and Johansson, 2008). Tight glucose control with insulin supply can also reduce neuropathy in DMT1 patients. However, the reduction of glucose levels is not enough to block the vicious cycle of oxidative stress, inflammation and cellular damage triggered by hyperglycaemia (Han et al., 2013).

- VASCULAR AND GROWTH FACTOR DEFICIENCY

Various studies report a major pathophysiological role of vascular and neurotrophic supply in diabetic neuropathy.

Maintaining adequate blood supply to nerves is crucial for maintaining nerve structure and function (Han et al., 2013). Deficiency in the nerve blood perfusion resulting in ischemic hypoxia largely contributes to pathogenic mechanisms of diabetic neuropathy as it determines malnourishment of nerve and thus neuronal dysfunction.

Pathologies of nervous and vascular system are highly intertwined in diabetes. As the disease progresses, neuronal dysfunctions correlate closely with the development of vascular abnormalities, such as capillary basement membrane thickening and endothelial hyperplasia, which contribute to diminished oxygen tension and hypoxia (Duby et al., 2004). Impaired vasodilation in diabetic epineurial arterioles (caused by ROS) decreases nerve conduction velocity.

Neurotrophic support has also an important role in diabetic neuropathy. Reduced neurotrophic supply in experimental diabetes was found to contribute to nerve malnourishment and neuronal dysfunction (Ekberg and Johansson, 2008).

Many growth factors exert both neurotrophic and angiogenic effects. In ischemic tissues, VEGF induces angiogenesis by stimulating the proliferation and migration of endothelial, thus improving tissue ischemia. It also promotes axonal outgrowth and survival of neurons and Schwann cells in DRG. Like VEGF, IGF induces vessel remodelling and has neurotrophic effect. It also stimulates Schwann cell mitogenesis and myelination. NGF, a well-known neurotrophic factor, promotes survival and differentiation of sensory and sympathetic neurons (Han et al., 2013). Moreover it provides neuroprotective and repair functions. In addition to these neurotrophic effects, NGF directly induces angiogenesis (Graiani et al., 2004).

- NEUROINFLAMMATION

Oxidative stress in combination with the activation of the classic metabolic pathways mentioned above, especially the MAPK signalling and the increased production of AGEs, can directly or indirectly initiate and progress the production of inflammatory mediators leading to neuroinflammation and thus nerve damage.

Activation of RAGE on microglia and macrophages initiates an inflammatory cascade through the activation of the transcriptional factor Nf- κ B, a potent inducer of the inflammatory processes (Yan et al., 1994). The activation of this transcriptional factor results in the up-regulation of genes encoding for pro-inflammatory cytokines (IL-1 β , TNF- α and IL-6) and chemokines, induction of neuronal apoptosis, and suppression of antioxidant genes with a consequent weakening of the innate antioxidant defence (Ganesh et al., 2013).

A persistent hyperglycaemic state also induces neuroinflammation by affecting the structural features of neurons; the glycosylation of myelin proteins alters their antigenicity and causes the infiltration of monocytes, macrophages, neutrophils from

the blood circulation as well as the activation of glia cells of the nervous system (King, 2001; Shi et al., 2013).

Moreover, NADP depletion mediated by PPAR overactivation leads to bioenergetic failure driving the cells towards necrosis. This mechanism contributes to neuroinflammation as the release of cellular debris by necrotic cells determines the recruitment of further inflammatory cells to injury site which enhance the local inflammatory response (Szabò, 2003).

Inflammatory cytokines released by either resident or infiltrating cells mediate damages to myelin sheaths and increase nerve excitability leading to edema and neuroinflammation. Moreover, inflammatory cells have a vicious positive feedback loop for increasing further production of inflammatory mediators thus potentiating nerve derangement (Sandireddy et al., 2014).

Hypoxia and ischemia created in diabetes aggravate the neuroinflammation by inducing iNOS activity, which is responsible for the release of nitric oxide (NO), another important mediator of inflammation.

2. 4 MURINE MODELS OF NEUROPATHIC PAIN

Although intensive researches over the last decades have contributed to make light of some of molecules, receptors, channels and pathways mainly associated to pathological pain, a clear comprehension of the mechanisms underlying the development and maintenance of neuropathic pain is unfortunately still far. Important advances in the study of neuropathic pain have been achieved using animal models, excellent systems to simulate the clinical pain conditions observed in human and to test novel therapeutic agents for contrasting this intractable pain. Even if most of animal models of neuropathic pain were initially generated in rats as preferred species, now the majority of pain models have been transposed in mice for the availability of genetically characterized or manipulated inbred strains in which specific proteins or signal transduction components have been altered throughout genetic knockout technology (Colleoni and Sacerdote, 2010).

According to the huge etiology of human painful neuropathy different types of well-characterized animal models have been developed over time in order to resemble as closely as possible the heterogeneity of the pain manifestations.

2.4.1 PERIPHERAL NERVE INJURY MODELS

Many animal models have been created to emulate human painful neuropathy, most of which are based on surgical procedures at or near the sciatic nerve for ease of access and its relative large size. Peripheral nerve transection, resection, crushing,

loose or tight ligation and cryoneurolysis are some of the most widely lesions induced in animals (Colleoni and Sacerdote, 2010).

Depending on the entity of nerve injury animals develop characteristic neuropathic behaviours. In neuroma model, a complete sectioning of the sciatic nerve is used to replicate the human syndromes seen after an amputation (phantom limb) or after transection in an intact limb (anesthesia dolorosa) (Janicki and Parris, 1997). Following the injury, a neuroma develops at the proximal nerve stump, consisting of regenerative nerve sprouting in all direction (Wang and Wang, 2003). The resulting immediate interruption of nerve conduction causes in animals episodes of self-attack and mutilation (autotomy) of their denervated limb, that are widely assumed as signs of chronic spontaneous pain. In this model allodynia and hyperalgesia are not detectable. Other models are based on the partial nerve lesions and include the chronic constriction injury (CCI) of sciatic nerve, the sciatic nerve partial ligation (PNS) and the spinal nerve ligations (SNL). These procedures preserve some innervation functions, mimicking clinical manifestations that occur after nerve trauma in human, and produce similar pain behaviours with some variations in the magnitude (Colleoni and Sacerdote, 2010). Injured animals experience both allodynia and hyperalgesia that are two specific symptoms of human peripheral neuropathy; on the contrary the presence of autotomy is restricted to some mouse strains.

The chronic constriction injury procedure is performed through three or four loose ligatures around the mid-thigh of sciatic nerve and results in intraneural oedema, which strangulates the nerve, effectively axotomizing many but not all the nerve axons, Wallerian degeneration and epineural inflammation (Colleoni and Sacerdote, 2010). After surgery, animals exhibit thermal hyperalgesia and allodynia to mechanical stimuli as signs of spontaneous pain that include guarding, excessive grooming and limping of ipsilateral hind paw and avoidance of placing weight on the injury side (Wang and Wang, 2003). Evidence of pain sensation is detected 2 to 7 days after injury, reaching peak severity in 10-14 days and disappearing in about two months (Janicki and Parris, 1997).

CCI-induced lesion produces a series of morphological, electrophysiological and pathological changes around the ligated nerve. In this model the processes of change are divisible into three phases: a first inflammatory phase characterized by a consistent inflammatory cell infiltration associated to the disruption of perineurium, demyelination with phagocytosis and axonal degeneration; a second phase consisting of axonal sprouting and remyelination processes and a third last phase in which maturing myelination and interstitial fibrosis lead to a complete recovery of the damaged area (Bai et al., 1999).

PNS and SNL are two other peripheral nerve injury models largely used to resemble human causalgia. PNS model is produced in animal through the ligation of a part, in general $1/3$ - $1/2$, of sciatic nerve thickness, while the SNL consists of unilaterally and

thigh ligation around L5-L6 spinal nerves. These procedures induce long-lasting neuropathic behaviours. In comparison to CCI model, painful symptoms following SNL and PSN surgery persist for at least 4 months, in the case of SNL, and over 7 months for PSN.

A recent model of neuropathic pain is the spared nerve injury (SNI). This involves a lesion of two of the three terminal branches of the sciatic nerve, the tibial and the common peroneal nerves, leaving the remaining sural nerve intact (Decosterd and Woolf, 2000). Contrarily to CCI, PNS and SNL models, this procedure allows the comparison of difference in mechanical and thermal sensitivities of non-injured skin territories adjoining to the denervated areas (Jaggi et al., 2011). Variants of SNI model are produced through different combinations of nerve transection.

Another technique to produce nerve injury is the sciatic nerve freezing. The sciatic cryoneurolysis (SCN) model induces a reversible damage of the nerve offering the opportunity to study the transient nerve injury and the healing process.

Ligation, transection or cryoneurolysis models show little difference in pain behaviours in the first 7 days after injury (Wang and Wang, 2003).

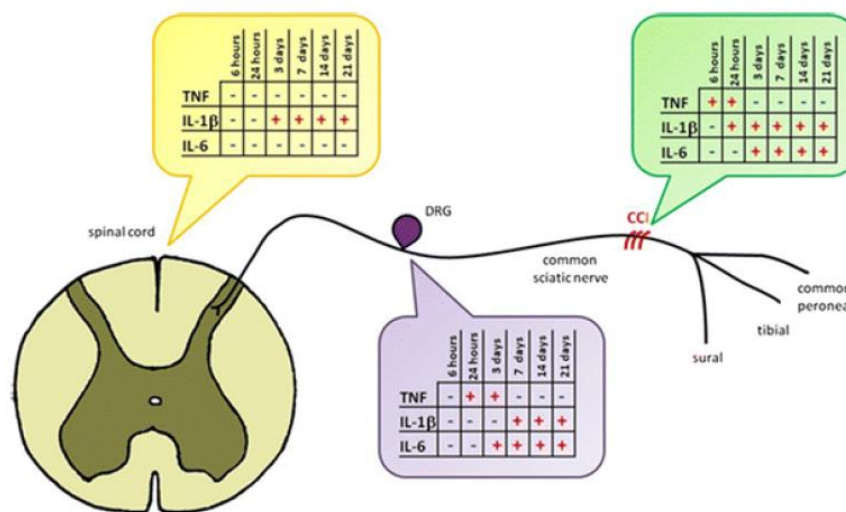


Figure 3 | Time course of the activation of the pro-inflammatory cytokines TNF, IL-1 β and IL-6 along somatosensory pathways (sciatic nerve, DRG and spinal cord) after nerve injury (CCI) in mouse. + indicates a significant increase of mRNA measured in the nervous tissue at different time points after CCI in comparison to sham operated animals – indicates no significant difference is present in comparison to sham operated animals. Figure taken from Sacerdote et al., 2013.

2.4.2 MODELS OF DIABETES-INDUCED PERIPHERAL NEUROPATHIC PAIN

Peripheral painful neuropathy often occurs as a consequence of metabolic (diabetes), viral (Herpes Zoster and HIV infection) and autoimmune (multiple sclerosis) diseases. Among these conditions, diabetes is the most frequent cause of neuropathic pain development.

Experimental models of diabetes exhibit behavioural responses similar to those present in diabetic neuropathic patients, including spontaneous pain, decrease in mechanical nociceptive thresholds and/or hypoalgesia characterized by decreased responses to mechanical and thermal stimuli (Colleoni and Sacerdote, 2010).

Murine models of diabetes are either chemically-induced or genetically determined. The induction of experimental diabetes using chemicals which selectively destroy pancreatic β -cells is the most commonly model used for the study of peripheral diabetic neuropathy (PDN).

Alloxan and streptozotocin (STZ), cytotoxic glucose analogues, are the most prominent diabetogenic chemicals in diabetes research (Lenzen, 2008). Both toxins selectively accumulate into β -cells via glucose transporter GLUT2. Although their cytotoxicity is achieved via different pathways, by the generation of reactive oxygen species in the case of alloxan and through DNA alkylation for streptozotocin, these chemicals produce severe DNA changes leading to a rapid destruction of β -cells with a consequent deficit in insulin production and rapid development of hyperglycaemia (Szkudelski, 2001).

In STZ-induced diabetic neuropathy model, single or repeated administrations of STZ produce in mice long-lasting thermal and mechanical hyperalgesia and cold, thermal and mechanical allodynia (Morita et al., 2008; Vareniuk et al., 2008). The main reason of STZ-induced β -cell death is the alkylation of the DNA (Elsner et al., 2002). Following its uptake into the β -cells, streptozotocin is split into its glucose and methylnitrosourea moiety and methyl groups are transferred from streptozotocin to the DNA molecule causing a damage, which ultimately results in the fragmentation of the DNA (Lenzen, 2008).

The metabolism of STZ occurring inside β -cells liberates also a toxic amount of nitric oxide (NO), which inhibiting aconitase enzyme activity induces a restriction of mitochondrial ATP production. (Welsh and Sandler, 1994).

Moreover, STZ-induced DNA damage activates the poly (ADP-ribose) polymerase (PARP). Its overstimulation leads to consumption of cellular NAD⁺ and further reduction of the ATP content (Heller et al., 1994; Lenzen, 2008). The depletion of the cellular energy stores ultimately results in β -cell necrosis with a subsequent inhibition of insulin synthesis and secretion (Nukatsuka et al., 1990). Protein glycosylation may be an additional damaging factor (Konrad and Kudlow, 2002). Depending on the choice of the experimental protocol STZ-induced diabetes involves different pathological

pathways. Direct β -cell toxicity is exerted by intraperitoneal (i.p.) injection of STZ (180-200 mg/kg bw), whereas i.p. injections of multiple low doses (40 mg/kg on five consecutive days) induce subtoxic effects on β -cell, leading to slow progressive hyperglycaemia, accompanied by lymphocytic infiltration of pancreatic islets, which resembles type 1 diabetes in human patients (Homs et al., 2011).

Due to the selectivity for the glucose transporter GLUT2, STZ induces toxic effects also on other organs expressing this transporter, particularly kidney and liver. As result animals exhibit over time a progressive deterioration of their general condition.

Most studies on the etiopathogenesis of type 1 diabetes (T1D) have focused on the non-obese diabetic (NOD) mouse model, which shares many genetic and immunological disease characteristics with human T1D (In't Veld, 2014). In this model spontaneous development of the autoimmune diabetes involves a long-term inflammatory process. Similarly to diabetic patients, in NOD mice neuropathic symptoms, including hyperalgesia, appear during the early inflammatory stage of the disease and precede the onset of an evident hyperglycaemic state (Colleoni and Sacerdote, 2010). Other animal models that exhibit long-term complications of diabetes include transgenic insulin resistant ob/ob and db/db mice. These animals develop obesity and type 2 diabetes as a result of spontaneous genetic mutations that cause a decrease in functional leptin (ob/ob) or its receptor (db/db) (Colleoni and Sacerdote, 2010).

2.5 THE PROKINETICIN SYSTEM

Prokineticins (PKs) are a family of secreted peptides highly preserved throughout evolution, from invertebrates (crayfish, shrimp) to various species of mammals, including human.

The first members of this family to be identified 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 skin secretion of the frog *Bombina variegata* (8 kDa) (Mollay et al., 1999). Bv8-like proteins were subsequently recognized in skin secretion of other amphibians, in reptiles, fishes and mammals. In mammals two Bv8 homologs were identified: the prokineticin 1 (PK1 or endocrine gland-derived vascular endothelial growth factor, EG-VEGF) and the prokineticin 2 (PK2 or mammalian-Bv8) (Negri et al., 2007).

Prokineticins are involved in a large spectrum of functions in the organism. They were originally determined as potent agents mediating gut motility in the digestive system, but were later shown to promote angiogenesis in steroidogenic glands, heart and reproductive organs (Ngan and Tam, 2008). Moreover over the last decades it was demonstrated that numerous other biological activities are associated to prokineticins,

such as neurogenesis, circadian rhythms, nociception, hematopoiesis as well as inflammatory and immunomodulatory processes (Negri et al., 2007). In addition, these proteins are implicated in pathologies affecting the reproductive and nervous system, cardiomyopathy and tumorigenesis (Ngan and Tam, 2008).

2.5.1 PROKINETICINS AND PROKINETICIN RECEPTORS: gene sequence, protein structure and anatomical localization

Mammalian prokineticins PK1 and PK2 are encoded by distinct genes at different localization. PROK1 gene 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 PROK2 gene is located on human chromosome 3p21.1 and mouse chromosome 6 (Jilek et al., 2000) and consists of four exons which give rise to two mature proteins: PK2 of 81 aminoacids and a splice variant with 21 additional basic aminoacids called PK2L of 102 aminoacids, which is known to be processed by protease cleavage into a smaller active peptide called PK2L β (Chen et al., 2005). However, the biological function of the long isoform PK2L and PK2L β has not yet been elucidated.

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 elevated structural identity, particularly in the N-terminal and central regions. All these proteins in fact exhibit an identical amino terminal sequence, AVITGA (Ala, Val, Ile, Thr, Gly, Ala), which is critical for receptor recognition and the biological activity (Bullock et al., 2004; Negri et al., 2005).

The presence of this distinctive preserved domain led Kaser et al. to define them the "AVIT proteins" in order to group in a unique family (AVIT family) the prokineticins and their non-mammalian orthologs.

The N-terminal hexapeptide sequence is essential for the function of PK2. Deletions, insertions and substitution mutations (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.

Another common features of these proteins are 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 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 molecules (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 impressive array of organs including brain, ovary, testis, placenta, adrenal cortex, intestinal tract, heart 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 central nervous system and non-steroidogenic cells of testis (Ferrara et al., 2004; Cheng et al., 2005).

Contrary to low expression of PK1 detected in brain, PK2 is abundantly expressed in this organ, particularly in suprachiasmatic nucleus and the olfactory bulb (Cheng et al., 2002; Ng et al., 2005). Almost simultaneously with the discovery of the prokineticins, three independent research groups identified in mammals two closely related G protein receptors for Bv8/PKs, prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2) (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). Prokineticin receptors belong to the neuropeptide Y (NPY) receptor class, have an overall identity in their aminoacid sequences of 85% with most 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 PKR1 maps to human chromosome region 2q14 and mouse chromosome 6, while that for PKR2 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 PKR1 and PKR2 revealed a general non-selectivity of all prokineticins for either receptors, except MIT-1 and PK2L β that display a clear selectivity for PKR2 and PKR1, respectively (Negri et al., 2007). The affinity of PK1 and PK2 for their receptors are in similar range, although PK2 displays a moderately higher affinity for both receptors: the K_d (nM) values for PK1 and PK2 binding to PKR1 are 12.3 ± 4.2 and 1.4 ± 0.5 , respectively, the K_d (nM) values for PK1 and PK2 binding to PKR2 are 1.8 ± 0.1 and 2.0 ± 0.7 , respectively (Lin et al., 2002a; Soga et al., 2002; Cheng et al., 2005; Maldonado-Perez et al., 2007).

All prokineticins activate PKR1 and PKR2 in the nanomolar concentrations. PKRs 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 al., 2003; Maldonado-Perez et al., 2007; Ngam and Tam, 2008). Multiple intracellular pathways are triggered by PKRs. One of the major signalling mechanisms is the intracellular calcium mobilization via protein G_q activation. PKRs/G_q interaction by activating phospholipase C β (PLC β) promotes the formation of inositol triphosphate and the consequent calcium release from intracellular stores leading to altered cell activity (Lin et al., 2002b).

Intracellular calcium stimulation also 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 PKRs increase 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, PKRs mediate the phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK), serine/threonine kinase Akt and cAMP accumulation, respectively (Chen et al., 2005).

PKRs are widely distributed in the organism and frequently, tissues with high levels of PKR1 exhibit low to detectable levels of PKR2 and vice versa (Martin et al., 2011).

PKR1 is mainly, but not exclusively, diffused 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 al., 2002; Cheng et al., 2006). PKRs 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). In CNS, PKR2 is prevalently expressed in neurons, whereas PKR1 is mostly expressed in glia cells, as indicate by analysis on primary cultured neurons, astrocytes and microglia prepared from mouse cerebrum (Koyama et al., 2006).

The distinct expression patterns of the receptors and ligands gives reasons for the large spectrum of biological activities exerted by prokineticins. Differential G-protein expression pattern and multiple G-protein coupling of the receptors furthermore increase the functional complexity of the system (Ngam and Tam, 2008).

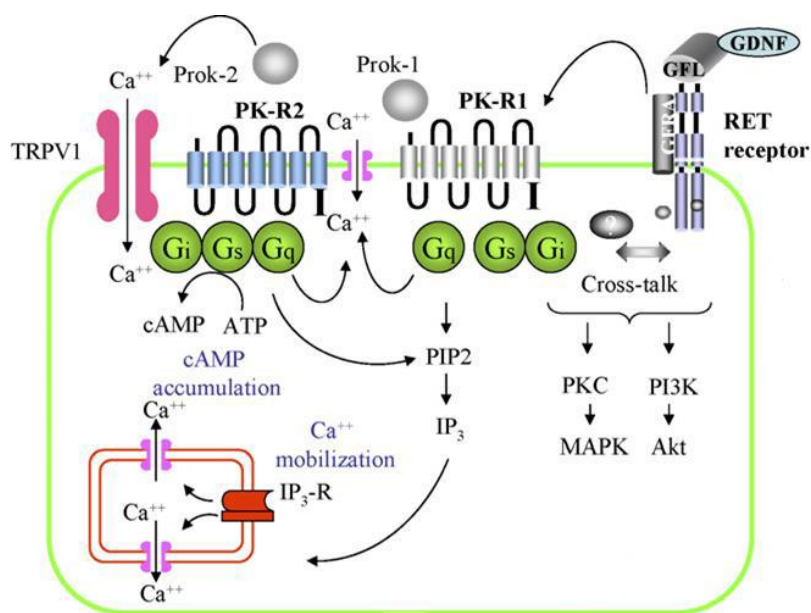


Figure 4 | **Prokineticin signalling.** Figure taken from Ngan and Tam, 2008.

2.5.2 BIOLOGICAL FUNCTIONS OF PROKINETICINS

○ *Gastro-intestinal motility*

Prokineticins are firstly identified in the gastrointestinal tract as endogenous regulatory agents mediating gastrointestinal motility by their ability to contract isolated guinea pig ileum and promote relaxation of colon (Li et al., 2001).

The role of PKs in gastric and colon contractility was later demonstrated also in rodents. In murine proximal colon PK1 was found to suppress spontaneous giant contractions of the circular muscle via 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*

PK2 and PKR2 are widely expressed in CNS. In situ hybridization studies have demonstrated 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), a region where neurogenesis persists also in adulthood (Melchiorri et al., 2001).

Expression analysis for prokineticin receptors in rodents at various ages has furthermore shown differential anatomical and temporal localization of PKRs within the CNS. One day after birth PKR2 is strongly expressed in the olfactory

bulb, neuroepithelium, striatum, hippocampus, thalamic and hypothalamic areas, amygdala and cortex of rat, whereas PKR1 expression is restricted in the cortex; in the brain of adult rat only PKR2 is still expressed. Both receptors are instead 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/PKR2 signalling pair has a critical role in the development of olfactory bulb. PK2 and PKR2 deficient mice display hypoplasia and defects in the architecture of OB (Matsumoto et al., 2006; Negri et al., 2007). PKR2 null mice also show severe atrophy of the reproductive system, pathological change which resembles the clinical manifestations of Kallmann syndrome, human developmental disease characterized by hypogonadotropic hypogonadism, ascribed 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 drives daily rhythms of diverse physiological functions and behaviours, including the sleep/wake cycle, blood pressure and energy metabolism (Reppert and Weaver, 2002).

Mapping the distribution of prokineticins and cognate receptors in mouse brain has revealed that PK2 and PKR2 are abundantly expressed in SNC indicating a potential regulator function for PK2/PKR2 signalling in the circadian clock. Prokineticins levels in SCN display dramatic circadian rhythmicity under light/dark; hypothalamic PK2 mRNA is high during the day, while very low levels are detected in the dark phase (Cheng et al., 2002). Moreover, high levels of PKR2 are detected in the dorsal medial nucleus (DMN), and the paraventricular thalamic nuclei (PVT), brain structures known to regulate circadian activity. Contrarily to PK2 expression, the levels of PKR2 show no significant diurnal alteration (Matsumoto et al., 2006).

The involvement of the PKs system in circadian rhythms regulation is supported by studies performed in transgenic animal models. It was in fact demonstrated that PK2-deficient mice exhibit reduced rhythmicity for a variety of physiological and behaviour parameters, including sleep/wake cycle, activity, body temperature, circulating glucocorticoid, glucose levels and the expression of peripheral clock

genes (Li et al., 2006). PKR2 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. 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, reproductive system and tumorigenesis

The angiogenic activity of prokineticins is well documented. 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 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 PKR2 on endothelial cells, suggested 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 PKRs widely expressed in endothelial cells from different tissue vasculatures. The activation of PKR1 enhances cell survival and proliferation while PKR2 is mostly implicated in regulating endothelial cell permeability (LeCouter et al., 2003a; Lin et al., 2002b; Kisliouk et al., 2003).

PK2/PKR1 pair signalling not only promotes the growth of capillary endothelia, but also modulates the cardiomyocyte survival, inducing vessel-like formation and protecting cardiac cells against oxidative stress (Urayama et al., 2007).

PKs and PKRs are predominantly expressed in reproductive system. 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 (Maldonado-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 absent (Ferrara et al., 2004). On the contrary a strong expression of PK1 was found in the endocrine component of placenta, the syncytiotrophoblast layer, where PK1 could act as a novel placental growth factor for trophoblast differentiation (Maldonado-Perez et al., 2007). PK1 is also present in the ovary showing a dynamic pattern of expression during the ovulatory cycle and pregnancy (Ferrara et al., 2004). In addition, in

uterus PK1 was found to facilitate implantation by increasing microvascular permeability of the endometrium (Ngam et al., 2006).

On the other hand, aberrant PKs signalling results in hyperplasia and hypervascularity in various tissues and is associated to the development of polycystic ovarian syndrome, neuroblastoma, colorectal, prostate and testicular cancers (Maldonato-Perez et al., 2007; Ngam and Tam, 2008). Clear evidences also indicate a potent role of PK2 released by infiltrating myeloid cells in promoting tumor angiogenesis (Shojaei et al., 2007).

2.5.2.1 ROLE OF PROKINETICINS IN PAIN

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

PK2 enhances pain sensitivity through the activation of PKR1 and PKR2, widely expressed in anatomical stations associated 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 very low doses of PK2 in rodents caused strong and localized hyperalgesia by decreasing 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 attributed to a local action of PK2 on nociceptors, because PK2 injected intraplantary 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 probably results from a central action of PK2 as it is not seen with the local 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-Gen 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).

PKR1 and PKR2 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 nociceptor 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 PKC ϵ 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 PKR1-positive DRG neurons express TRPV1 (Hu et al., 2006). The critical role of TRPV1 in the downstream signalling of PKR1 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 PKs system in central modulation of pain is documented by DeNovellis and co-workers. This study reports that intra-periaqueductal grey (PAG) administration of PK2 exerts a pro-nociceptive action increasing the intrinsic GABAergic tone which, in turn, is responsible for the inhibition of PAG anti-nociceptive output neurons impinging on RVM neurons (DeNovellis et al., 2007).

Recently it was also highlighted a crucial role for PK2 in mediating inflammatory hyperalgesia (Giannini et al., 2009). In animal model of inflammatory pain induced by complete Freund's adjuvant (CFA), a strongly increase of PK2 in granulocytes and macrophages correlates with the development and duration of pain.

Evidence for a direct involvement of PKs/PKR pair signalling in modulating pain processing also comes from studies in PK2-, PKR1- or PKR2- deficient mice (Negri et al., 2006; Hu et al., 2006). Animals lacking the PKRs or PK2 exhibit impaired pain perception to various stimuli, including noxious heat, mechanical stimuli and capsaicin. PKR1-deficient mice also display an impaired development of hyperalgesia after tissue injury.

2.5.2.2 FUNCTION OF PROKINETICINS 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).

Coupling to cognate receptors, prokineticin 2 was found to modulate 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 PK1 supports the differentiation of mouse and human bone marrow cells into monocyte/macrophage lineage (Dorsch et al., 2005), and a similar effect of PK2 was observed on monocyte lineage (LeCouter et al., 2004).

Moreover, prokineticins were shown to regulate the functioning of immune cells. PK1 and PK2 are in fact highly expressed on mature blood cells types. PK2 expression is particularly associated to monocytes, neutrophils and dendritic cells, and this protein is

released in large quantity by neutrophils at site on inflammation where it stimulates migration of monocytes (LeCouter et al., 2004).

PK1 promotes monocyte 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 reported the ability of PK2 to induce macrophage migration at very low concentrations and to acquire a pro-inflammatory phenotype significantly increasing the LPS-induced production of the pro-inflammatory cytokines IL-1 β and IL-12, while decreasing that of 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 exquisitely mediate by the activation of PKR1 because they lack in macrophages and spleen lymphocytes from PKR1 deficient mouse, 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 regulation of prokineticin and prokineticin receptors within immune cells (Monnier and Samson, 2008). However, PK2 transcription was recently shown 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 unable to block the actions of PK2, but rather inhibition of the G_q protein pathway blocked the secretion of cytokines mediated by PK2 (Martucci et al., 2006).

Prokineticins were also found to be involved in inflammatory diseases and tumours. 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 associated with infiltrating 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 PKRs in inflammatory pain as in mice lacking PKR1 or PKR2 a significant less inflammation-induced hyperalgesia was observed in comparison with WT mice. However, the inflammation-induced up-regulation of PK2 was significantly less in PKR1 deficient mice than in WT and PKR2 null mice, once again demonstrating a specific involvement of PKR1 in setting the

enhanced PK2 levels during inflammation (Giannini et al., 2009; Negri and Lattanzi, 2011).

It was supposed that the activation of PKR1 present 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).

2.5.3 PROKINETICIN RECEPTOR ANTAGONISTS

Considering the involvement of the PKs system as peripheral and central modulator in a wide spectrum of biological functions and pathological conditions in various tissues, the development of effective PKR antagonists may be useful in the treatment of various disease states.

Interestingly PK2 was found to be a potent pro-nociceptive/pro-inflammatory agents regulating pain processing. It follows that the antagonism of PKs signalling could also represent a novel promising approach to control different kinds of pain.

The identification of the structural determinants required for receptor binding and hyperalgesic activity of PKs is mandatory for the design of PKR antagonists (Balboni et al., 2008). As previously described, the highly conserved amino terminal sequence AVITGA and the tryptophan (Trp) residue in position 24 in all members of the PKs family are essential for their biological activity.

As suggested by Miele et co-workers, AVIT proteins could interact with PKRs by orienting the protein region that comprises 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 PKR antagonist 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 PKR2, 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 PKR antagonists, triazine-guanidine derivatives, have been synthesized and developed.

The "lead compound" of triazine antagonists is the molecule named PC1. 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 (Balboni et al., 2008).

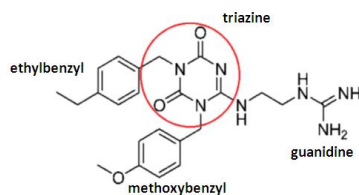


Figure 5 | **PC1 chemical structure.**
Red circle indicates triazine group.

Results from binding assay demonstrated that PC1 is a PKR1-preferring ligand. This molecule displays in fact an affinity about 70-fold higher for PKR1 ($K_i=22$ nM) than for PKR2 ($K_i=1610$ nM).

In vitro studies revealed a clear antagonist activity of PC1 as it was found to inhibit Bv8-induced intracellular calcium mobilization in PKR1- and PKR2-transfected CHO cells (Balboni et al., 2008). In vivo studies furthermore demonstrated the efficacy of PC1 to selectively antagonize Bv8-induced hyperalgesia and capsaicin-induced thermal hypersensitivity, suggesting that it may prevent activation of PKRs and TRPV1 by their endogenous ligands (Negri and Lattanzi, 2012).

In CFA-induced inflammatory pain model, systemic injections of PC1 (from 20 to 150 $\mu\text{g}/\text{kg}$, s.c.) reduced hyperalgesia in dose-dependent manner, completely abolishing it at the dose of 150 $\mu\text{g}/\text{kg}$ (Giannini et al., 2009).

3. AIM OF THE WORK

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 recently discovered family of chemokine-like proteins, the prokineticins (PKs).

Prokineticin 2 (PK2) belongs to this new family of chemokines and was found to have a pivotal role in pain transmission and immunomodulation acting on its receptors PKR1 and PKR2 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 threshold 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. The stimulation of PKR1 induces a pro-inflammatory phenotype of the macrophage, activating this cell 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 PKR1 and PKR2 as well as PK2 are expressed in neurons, glia and immune cells and that this system is involved in nociception and immunoregulation, PK2/PKR pair might exerts a critical role in chronic pain transmission (Koyama et al., 2006).

On these premises, the aim of my PhD project was to investigate the role of the PKs system in the development of neuropathic pain in order to identify new targets for the development of novel modality to control this kind of pain.

The involvement of this system was investigated in two widely accepted mouse models of neuropathic pain, i.e. a mononeuropathy induced by the chronic constriction injury (CCI) of sciatic nerve and a diabetic polyneuropathy induced by the injection of a pancreatic β cell toxin, streptozotocin (STZ).

In order to understand if PK2, PKR1 and PKR2 activities were necessary for the onset, maintenance or resolution of neuropathic pain, mice were chronically treated with the PKR antagonist PC1, which was proved to be effective in inflammatory pain treatment (Giannini et al., 2009). Different approaches and complementary methods (behavioural, biochemical and histochemical analysis) were used to prove the existence of a correlation between the presence of pain symptoms and the activation of the prokineticin system.

The CCI model was used in the first part of the study in order to characterize the PKs system in the main anatomical stations associated to neuropathic pain processing. The

efficacy of PC1 to counteract CCI-induced neuropathic painful symptoms, i.e. thermal hyperalgesia and mechanical allodynia, was tested and at the end of the chronic treatment with the antagonist the variations in pain behavior were evaluated and correlated with (i) the changes in mRNA expression and protein distribution of PK2 and PKRs in spinal cord and in the sciatic nerve by Real-Time PCR and immunocytochemistry (ii) the changes in mRNA expression and protein content of the pro-inflammatory cytokine IL-1 β and the anti-inflammatory IL-10 in spinal cord and in the sciatic nerve by Real-Time and ELISA analysis.

Since one of the major cause of neuropathic pain in human is the presence of diabetes, the next step was to investigate the role of the PKs system in experimental diabetic model induced by STZ injection, i.e. the painful peripheral diabetic neuropathy model. In order to deepen knowledge about the timing of activation and the specific role of the PKs system in neuropathic pain evolution in this model mice were chronically treated with the PKR antagonist PC1 at different time points from diabetes induction, i.e. either when animal exhibited an overt neuropathic pain (therapeutic treatment) or in mice not already neuropathic (preventive protocol) .

The efficacy of PC1 to counteract STZ-induced neuropathic painful symptoms, i.e. mechanical allodynia, was tested and at the end of the chronic treatments with the antagonist the variations in pain behavior were evaluated and correlated with (i) the changes in mRNA expression of PK2 and PKRs in spinal cord and in the sciatic nerve by Real-Time PCR (ii) the changes in protein content of glutamate AMPA and NMDA receptor subunits in spinal cord by Western blot analysis, and (iii) the changes in mRNA expression and protein content of the pro-inflammatory cytokine IL-1 β and the anti-inflammatory IL-10 in spinal cord and in the sciatic nerve by Real-Time and ELISA analysis.

As inflammation and alteration in immune system are known to be underpinning mechanisms in the pathophysiology of type 1 diabetes as well as of its complications, including neuroinflammation (Agrawal and Kant, 2014), the efficacy of chronic PC1 treatment to counteract the hyperglycaemic state of animals or to reduce the peripheral inflammatory component was also investigated, evaluating (i) the blood glucose concentration and plasmatic insulin levels by using a glucometer and ELISA method, respectively, (ii) the protein content of the pro-inflammatory cytokine IL-1 β and the anti-inflammatory IL-10 in pancreas by ELISA analysis, and (iii) the functioning of peripheral immune system by the measurements of cytokine production by peritoneal macrophages and spleen lymphocytes from diabetic mice treated with either PC1 or saline, by ELISA.

4.MATERIALS and METHODS

4.1 ANIMALS

All experiments were performed in accordance with the Italian Ministry of Health guidelines (DL 116/92 and DL111/94-B) and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Painful neuropathy was induced in C57BL/6J male mice weighing 20-25 g 9 weeks old (Harlan Laboratories, Italy). Animals were housed under controlled conditions with light/dark cycles of 12 hours, temperature of 22 ± 2 °C, humidity of $55 \pm 10\%$, food and water *ad libitum* and were acclimatized to the new environment for at least one week before being used.

4.2 INDUCTION OF PAINFUL NEUROPATHY

4.2.1 PERIPHERAL NERVE INJURY MODEL

Peripheral painful mononeuropathy was induced in mice according to Chronic Constriction Injury (CCI) model originally described by Bennet and Xie (1987) for rats.

Mice were anesthetized with sodium pentobarbital (i.p. 60 mg/kg, 0.1 ml/10 g) and an incision at the level of the right mid thigh, parallel to sciatic nerve, was made. The right common sciatic nerve was then exposed, separated from surrounding connective tissue and, taking care to preserve epineural circulation, three ligatures with sutures thread (4/0 chromic silk, Ethicon, Belgium) were loosely tied around it until a brief twitch in the respective hind paw was elicited. Ligatures were made upstream of the nerve trifurcation at about 1.0-2.0 mm of interval spacing one from the other. To avoid possible local infections, the wound was treated with neomycin (Boehringer Ingelheim, Italy) and closed with suture thread (3/0 chromic silk, Ethicon, Belgium).

Sham-operated animals, i.e. mice subjected to the same surgery procedure of CCI animals except that sciatic nerve was not tied, were used as control.

Since it was previously demonstrated that three days after surgery, the nociceptive thresholds and immune parameters of sham-operated animals were comparable to values of animals not subjected to any surgical procedure, naïve mice were not included in this study in order to reduce the number of animals used (Sacerdote et al., 2008).

4.2.2 DIABETES-INDUCED NEUROPATHIC PAIN MODEL

Type 1 diabetes was induced in mice through chemical pancreatectomy by a single (200 mg/kg, 0.1 ml/10 g) or repeated (MLD, 80 mg/kg for three consecutive days, 0.1

ml/10 g) administrations of streptozotocin (STZ) (Sigma Aldrich, Italy) intraperitoneally (i.p.), freshly prepared in citrate buffer 0.1 M, pH 4.55. Control mice were i.p. injected with citrate buffer (Noh et al., 2013).

The development of diabetes was monitored 7 days after STZ administrations evaluating the blood glucose levels of each animal. Blood samples were obtained from a small prick on the caudal vein of the animals and glucose concentration was assessed using a glucometer (GLUCOCARD G⁺ meter, A. Menarini diagnostics, Italy). Only the animals with blood glucose values above 250 mg/dl were considered diabetic; mice with blood glucose values inferior to this concentration were excluded from the study. Blood glucose levels and mouse body weight were monitored over the entire length of the experimental studies.

4.3 EXPERIMENTAL DESIGN

Various sets of experiments are performed. Schematic experimental designs used for the study of the PKs system in neuropathic pain induced by CCI and diabetes are depicted in figure 6.

4.3.1 TREATMENT

The PKR antagonist PC1, a triazine-guanidine compound (Balboni et al., 2008), was dissolved in sterile saline solution and used at the dose of 150 µg/kg, a dose which was clearly demonstrated to be effective in alleviating inflammatory pain (Giannini et al., 2009). Animals were subcutaneously injected with the PKR antagonist PC1 (0.1 ml/10 g) twice-daily for different consecutive days, depending on experimental animal model used. Control animals were injected with an equal amount of sterile saline solution.

Mice subjected to CCI surgery were treated with PC1 or saline for 7 days starting from the third postoperative day, time point corresponding to full painful symptom development (figure 6, panel A). STZ-treated animals received PC1 or saline administrations for 14 days starting either 21 days after diabetes induction, when they were already hyperglycaemic and neuropathic (therapeutic protocol) (figure 6, panel B, a), or at the same time of STZ administrations (day 0), i.e. when hyperglycaemia and mechanical allodynia were not yet developed (preventive protocol) (figure 6, panel B, b).

PERIPHERAL NERVE INJURY (CCI) MODEL

For these studies animals were randomly divided in four groups:

- Sham-false operated animals treated with saline (Sham/saline)
- Sham-false operated animals treated with PC1 (Sham/PC1)

- CCI animals treated with saline (CCI/saline)
- CCI animals treated with PC1 (CCI/PC1)

DIABETES-INDUCED NEUROPATHIC PAIN (STZ) MODEL

For these studies animals were randomly divided in four groups:

- Control animals treated with saline (CTR/saline)
- Control animals treated with PC1 (CTR/PC1)
- STZ-injected animals treated with saline (STZ/saline)
- STZ-injected animals treated with PC1 (STZ/PC1)

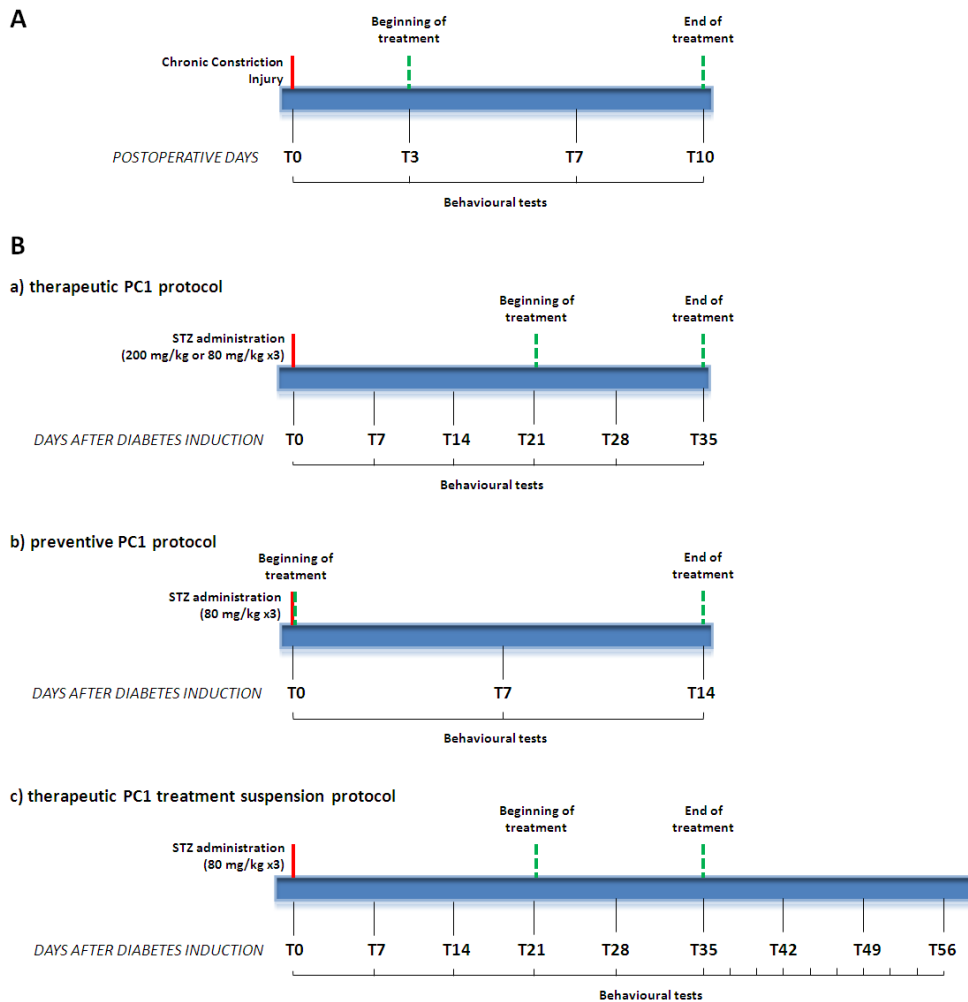


Figure 6- Experimental protocol schema. (A) therapeutic protocol used in peripheral nerve injury model; **(B)** therapeutic (**a** and **c**) and preventive (**b**) protocols used in diabetes-induced neuropathic pain model.

4.3.2 CHARACTERIZATION OF NOCICEPTIVE BEHAVIOUR

In order to characterize the nociceptive behaviour of neuropathic mice, two of the most frequent symptoms encountered in neuropathic patients were evaluated, i.e. thermal hyperalgesia and mechanical allodynia, monitoring over time the threshold responses of the animals to thermal and mechanical stimuli, respectively.

Behavioural testing was performed before neuropathic induction (T0), i.e. before CCI surgery and chemical pancreatectomy with STZ, to establish a baseline for comparisons with post-induction values, and at different successive times, as described below.

In the peripheral nerve injury model the responses to thermal and mechanical stimuli were assessed at T0 and at 3, 7 and 10 days after CCI surgery on the ipsilateral and controlateral hind paws of all mice (fig.6, panel A).

In the diabetes-induced neuropathic pain model the responses to mechanical stimuli were measured at T0 and, after diabetes induction, weekly for whole period of experimental study on both hind paws of all animals (fig.6, panel B). In order to investigate a potential long-lasting effect of the antagonist PC1 on nociceptive mechanical sensitivity, after 14 days of therapeutic PC1 administrations, the drug treatment was stopped, and behavioural testing was performed most frequently, every 2/3 days, until the disappearance of PC1 efficacy (56 days after STZ administration) (fig.6, panel B, c).

In all experiments, in order to avoid the evaluation of potential acute effect of the antagonist on nociceptive thresholds, on days of behavioural testing, the PC1 administration was immediately performed after the pain behaviour assessment.

The effect of a single bolus of PC1 was instead studied in STZ-injected mice 21 days after diabetes induction evaluating the responses to mechanical stimuli 30, 60, 120 and 240 minutes after PC1 administration.

4.3.3 TISSUE AND CELL SAMPLING

PERIPHERAL NERVE INJURY (CCI) MODEL

- Spinal cord and sciatic nerve collection for biochemical analysis

At the end of different sets of studies, spinal cord and injured-right sciatic nerve were dissected from CCI neuropathic mice treated with either saline or PC1 and their respective controls, immediately frozen in liquid nitrogen and conserved at -80°C until use.

The mRNA expression of PK2, PKR1 and PKR2 and the mRNA levels and the protein amount of IL-1 β and IL-10 in spinal cord and sciatic nerve were evaluated on the day after the last therapeutic PC1 administration in animals killed 10 days after CCI surgery.

- Spinal cord and sciatic nerve collection for PK2, PKR1 and PKR2 immunohistochemical analysis

At the end of chronic PC1 treatment, 10 days after CCI surgery, 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. Spinal cord (L4-L5 region) and injured-right sciatic nerve were dissected from animals, post-fixed overnight in the same fixative at 4°C and after three washes in PBS, finally cryo-protected in 30% sucrose at 4°C.

Subsequently, the sciatic nerves were embedded in paraffin while spinal cord samples were maintained in 30% sucrose at 4°C until use.

DIABETES-INDUCED NEUROPATHIC PAIN (STZ) MODEL

- Spinal cord and sciatic nerve collection for biochemical analysis

At the end of different sets of studies, spinal cord and sciatic nerves were dissected from diabetic neuropathic mice treated with either saline or PC1 and their respective controls, immediately frozen in liquid nitrogen and conserved at -80°C until use.

The mRNA expression of PK2, PKR1 and PKR2 and the mRNA levels and the protein amount of IL-1 β and IL-10 in spinal cord and sciatic nerves were evaluated on the day after the last therapeutic PC1 administration in animals killed 35 days after MLD-STZ injections. The mRNA expression of PK2 was also evaluated in spinal cord of mice killed 7 or 14 days after MLD-STZ injections, i.e. after only 7 days or at the end of preventive PC1 treatment respectively, and 21 days after the discontinuation of therapeutic PC1 administrations, corresponding to 56 days after MLD-STZ injections.

Protein content of glutamate NMDA and AMPA receptor subunits in spinal cord of neuropathic mice was evaluated on the day after the last preventive PC1 administration in animals sacrificed 14 days after MLD-STZ injection.

- Pancreas and plasma collection for biochemical analysis

At the end of preventive PC1 administrations, 14 days after diabetes induction, MLD-STZ injected mice treated with either PC1 or saline and respective controls was sacrificed for pancreatic tissue isolation. Pancreas were rapidly removed and immediately frozen in liquid nitrogen. Samples were conserved at -80 °C until IL-1 β and IL-10 protein content assay.

Before proceeding with tissue removal, a small aliquot of blood from fasted non-diabetic control and MLD-STZ injected mice treated either with PC1 or saline was collected in heparinized tubes for plasmatic insulin dosage.

Plasma separation was carried out by centrifuging the whole blood samples at 10000 rpm for 30 minutes at 4 °C. Samples were then stored at -20 °C until plasmatic insulin assay.

- Macrophages purification and stimulation for cytokine assay

Macrophage collection was performed at the end of both preventive and therapeutic PC1 treatment, 14 and 35 days after MLD-STZ injections, respectively. At these time points mice were killed and peritoneal macrophagic cells (PECs) were harvested in RPMI 1640 medium (collection medium) (Sigma-Aldrich) plus 10% FCS. Cell viability was checked by the Trypan blue exclusion test. Turk solution was used to discriminate nuclei and on the basis of their morphology, cells were counted.

The amount of cells recovered by a single mouse was very low as animals were not subjected to any macrophages elicitation. Hence, the cells obtained from mice belonging to the same treatment groups were pooled.

PECs were diluted in collection medium at the final concentration of 1×10^6 /ml, and 1 ml/well aliquots were dispensed into 24-well culture plates.

Isolation and purification of macrophages were carried out by 2 hours of adherence. As previously reported (Martucci et al., 2007), this procedure produces a population of macrophages with a 90% purity. Non-adherent cells were removed, and adherent cells washed twice with PBS solution and incubated with or without 1 µg/ml LPS for IL-1β and IL-10 stimulation. The stimulus was added to the macrophage cultures in a final volume of 1 ml/well in RPMI 1640 plus 10% FCS, 1% glutamine, 2% streptomycin solution and 0,1% 2-mercaptoethanol (complete RPMI).

After 24 hours of culture at 37 °C in 5% CO₂ and 95% air, the supernatant was collected and stored frozen at -80 °C for cytokine evaluation.

- Spleen cells collection and stimulation for cytokine assay

Spleen cell collection was performed at the end of both preventive and therapeutic PC1 treatment, 14 and 35 days after MLD-STZ injections, respectively.

At these time points mice were killed and their spleens rapidly and aseptically removed. Splenocytes were spilled out from an incision on spleen cuticle made with 20-gauge needles, adjusted in 24-well plates at the final concentration of 5×10^6 cell/ml of culture medium (complete RPMI, i.e. RPMI 1640 supplemented with 10% FCS, 1% glutamine, 2% antibiotics and 0.1% 2-mercaptoethanol) and incubated at 37 °C in 5% CO₂ and 95% air with or without 10 µg/ml Concanavalin A (ConA) for Th1 and Th2 cytokine stimulation. The stimulus was added to the cell cultures in a final volume of 1 ml/well in complete RPMI.

After 24 (in the case of IFN- γ and IL-2) or 48 hours (in the case of IL-4 and IL-10) of culture, times of maximum cytokines release (Sacerdote et al., 2000; Martucci et al., 2007), the supernatant was collected and stored frozen at -80 °C for cytokine assay. In order to evaluate the Th1/Th2 balance, the IFN- γ /IL-4 ratio was calculated for each mouse.

4.4 NOCICEPTIVE BEHAVIOURAL TESTS

All behavioural tests were carried out in the morning in a quiet temperature-controlled room after a habituation period of 30 minutes that allows an “appropriate behavioural immobility” of the animals. The behavioural evaluations were always performed by researchers who were blind to treatments.

4.4.1 PLANTAR TEST: THERMAL HYPERALGESIA EVALUATION

Thermal hyperalgesia, an increase of pain sensitivity to thermal painful stimuli, was assessed according to the Hargreaves procedure, using the Plantar Test Apparatus (Ugo Basile, Italy) (Martucci et al., 2008).



The instrument basically consists of a movable infrared (I.R.) generator lodged into a cylindrical vessel of aluminium, placed below a glass pane upon which is supported a Perspex enclosure within which the researchers place the animals. Three compartments, further subdivided by wooden structures 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, helping the operator to carry out a rapid “testing” work. The instrument is also equipped with a controller that allows the setting of the I.R. rays intensity (100 mW/cm² for mouse) and the reading (in seconds) of the paw withdrawal latency (PWL) time.

After the acclimation period the operators place the movable I.R. generator under the glass pane at the level of the hind paw of the animals and the heat stimulation of their mid plantar surface is commenced by depressing a start key. When mice feel pain and withdraw their paw, the I.R. source switches off and the reaction time, i.e. PWL, recorded. The cut-off time was set at 22 seconds in order to prevent tissue damage. PWL was measured three times on both hind paws and the mean of the values was calculated.

4.4.2 VON FREY TEST: MECHANICAL ALLODYNIA EVALUATION

Mechanical allodynia, 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, Italy). This instrument consists of a moveable force actuator containing the Von Frey filament, placed below a metallic perforated platform upon which the researchers deposit the animals, whose mobility is restrained by a Perspex cage very similar to that provided for the Plantar Test Apparatus (see above). The moveable force actuator is a cylindrical vessel equipped with an adjustable angled-mirror in order to position the touch probe below the target area of the paw and star keys to actuate a vertical movement of the 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).

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 recorded. PWT was measured three times on both hind paws and the mean of the values was calculated.



4.5 BIOCHEMICAL EVALUATION

4.5.1 REAL TIME PCR

Total RNA was isolated from sciatic nerves and the lumbar spinal cords (L4-L6) using Trizol[®] reagent (Trifast Eurogold[®], Euroclone, Italy) according to manufacturer's instructions and re-suspended in 10-20 μ l of RNase-free water. To achieve an adequate quantity of RNA, sciatic nerves were previously pooled from two mice belonging to the same experimental group.

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. Total RNA concentration was then determined from sample absorbance value at 260 nm and an equal amount of RNA underwent to reverse-transcription (RT) using iScript[™] Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Italy). cDNA was synthesized from 1000 ng of total RNA in a final volume of 20 μ l and was used as template in Real Time PCR for mRNA analysis.

Real Time PCR was performed using ABI PRISM 7000 system (Applied Biosystems, Foster City, CA) and carried out in a final volume of 25 μ l consisting of 2 μ l of cDNA (corresponding to 100 ng of cDNA), 10 μ l of TaqMan Universal PCR Real Master Mix Rox (Eppendorf, Italy), 1.25 μ l of TaqMan probe/primers and 11.75 μ l of RNase-free water (Martucci et al, 2007). The reaction mixture without the cDNA was used as control. Specific TaqMan probe/primers for mouse Prokineticin 2 (Prok2 Mm01182450_g1), Prokineticin receptors (Prokr1 Mm00517546_m1; Prokr2 Mm00769571_m1), interleukins (IL-1 β Mm00434228_m1; IL-10 Mm00439616_m1) and glyceraldehydes-3-phosphate dehydrogenase (Gapdh Mm99999915_g1) were purchased from Applied Biosystems.

All PCR assays were performed in triplicate. Before using the $\Delta\Delta C_t$ method for relative quantification, we performed a validation experiment to demonstrate that the efficiencies of targets and reference were approximately equal.

The reaction conditions were as follows: 95 °C for 2 minutes (Initial Denaturation) followed by 45 cycles at 95 °C for 15 seconds (Cycled Template Denaturation) and at 60 °C for 60 seconds (Annealing and Extension). Relative quantification was performed using the comparative threshold method. Threshold cycle numbers (C_T) of the specific gene of interest and the endogenous control gene GAPDH were determined by ABI PRISM 7000 Sequence Detection System and the amount of target gene was evaluated using the following formula $2^{-\Delta\Delta C_T}$. Briefly, C_T value of the specific target was normalized to the respective C_T value of GAPDH subtracting GAPDH C_T to target gene C_T (ΔC_T), while $\Delta\Delta C_T$ was calculated subtracting the GAPDH C_T average of saline treated

sham (for CCI) or control (non-diabetic mice) group used as calibrator to ΔC_T value of every sample.

4.5.2 ENZYME-LINKED IMMUNO-SORBENT ASSAY (ELISA)

- *Tissue preparation*

Sciatic nerves pooled from two mice belonging to the same experimental group and spinal cord samples were homogenized in 0.3 ml of ice-cold phosphate-buffered saline (lysis buffer) containing a protease inhibitor cocktail (Roche Diagnostics, Italy). Pancreatic tissues were homogenized in 2 ml of the same lysis buffer. All samples were centrifuged at 13000 rpm for 15 at 4 °C. Supernatants were collected and used to measure IL-1 β and IL-10 levels and total protein content (Lowry's method).

As previously described (see paragraph 4.3.3), culture media of macrophages and spleen cells were used for evaluating the production of IL-1 β and IL-10, and IFN- γ , IL-2, IL-4 and IL-10, respectively.

- *Cytokine ELISA*

Cytokine concentration was determined by Enzyme-Linked Immuno-Sorbent Assay (ELISA) using ultra-sensitive ELISA kits according to the manufacturer's instruction. DuoSet[®] ELISA development system for mouse IL-1 β , IL-2, IFN- γ and IL-4 was purchased from R&D Systems (Minneapolis, USA) while mouse IL-10 ELISA Ready-SET-Go! from eBioscience (San Diego, CA). Sensitive of the method for IL-1 β , IL-2 and IL-4 was 15.625 pg/ml; for IFN- γ was 31.25 pg/ml; and for IL-10 32 pg/ml.

The principle of all assays is a quantitative sandwich enzyme immunoassay technique. Cytokine concentrations were determined by interpolation with standard curves assayed on individual plates and normalized to protein content in each sample.

- *Plasmatic insulin assay*

Plasmatic insulin dosage was performed using a specific ELISA kit provided by Mercodia (Uppsala, Sweden). Sensitive of the method was 0.2 μ g/L. The principle of assays is a quantitative sandwich enzyme immunoassay technique. Insulin concentrations was determined by interpolation with standard curves assayed on individual plate.

4.5.3 WESTERN BLOT

- *Homogenates preparation*

Lumbar spinal cords (L4-L6) from mice belonging to the same treatment groups, i.e. from non-diabetic controls animals and MLD-STZ injected mice treated with either PC1 preventive administrations or saline, were pooled.

Samples were homogenised using a potter in Tris-HCl buffer (50mM Tris, 120 mM NaCl, 5mM KCl, 2.5 mM, 1mM MgCl₂, pH 7), washed once by centrifugation (1h; 25000g) and then resuspended in the proper volume of the same buffer containing a 20 µg/ml mixture of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin.

Total protein content was evaluated by using BCA protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

- *Antibody production and characterization*

We used affinity-purified, subunit-specific polyclonal antibodies (Abs), produced in rabbit against peptides derived from the C-terminal (COOH), N-terminal (NH) of mouse and AMPAR GluA1 and GluA2/3 subunits. The Ab against the GluA2/3 subunit was directed against the C-terminus peptide (EGYNVYGIESVKI). The Ab against the GluA1 subunit was directed against the extracellular domain peptides (RTSDSRDHTRVDWKR) corresponding to aminoacids 253-267 (271-285 if numbered from the signal peptide), this region is not conserved in GluA2-4, nor Kainate and NMDAR. GluA1 and GluA2/3 sequences were the same as those reported by Chemicon International. The specificity of the affinity-purified Abs was previously tested by western blotting studies using cells transfected and non-transfected with GluA1 and GluA2/3. Our tests do not show crossreactivity between GluA1 and GluA2/3 Abs, as it has been reported in the specificity tests of Chemicon International.

Anti GluN1 (clone 54.1) was from BD Pharmigen, anti GluN2A (clone A3-2D10) was from Invitrogen, anti-GluN2B (clone N59/20) was from Antibodies Incorporated, anti-tubulin (clone B-5-1-2) was from Sigma-Aldrich and anti Na/K ATPase was described in (Pietrini et al., 1992).

- *Immunoblotting and densitometric quantification of Western blot bands*

The analysis of the GluR subunits by Western blotting was performed as described previously (Gotti et al., 2008). In brief, depending on the target subunit, 2.5, 5, or 10 µg of total homogenates samples were diluted 1:1 (v/v) with Laemmli buffer and then underwent SDS-PAGE using 7.5% acrylamide gel. After SDS-PAGE, the proteins were

electrophoretically transferred to nitrocellulose membranes with 0.45-mm pores (Schleicher and Schu"ll, Dassel, Germany). The blots were blocked overnight in 4% non-fat milk in Tris-buffered saline, washed in a buffer containing 4% nonfat milk and 0.3% Tween 20 in Tris-buffered saline, incubated for 2 h with the primary antibody at the following concentration (GluA1 and GluA2/3: 1–2.5 mg/ml; GluN1 1:1500; GluN2A 1:1000; GluN2B 1:600; Na/K ATPase 1:1000; tubulin 1:10000) and then incubated for 1h with the appropriate secondary antibody (anti-rabbit Ly-CorIRDye800RD: 1:10000 ; anti-mouse Ly-Cor IRDye680RD: 1:7500). Membranes were further washed in Tris-buffered saline and dried overnight at RT in darkness. The IR signal was measured through the IR scanner Odyssey CL220x - Infrared Imaging System.

The quantification of the signal intensity of the Western blot bands was performed with iStudio software. The optical density ratio was calculated by taking the optical density of the control as 100%. The values are the mean \pm SEM of 7-8 separate experiments for each antibody.

All the experiments were performed in the laboratory of Dr. Cecilia Gotti (CNR Institute of Neuroscience, Milan).

4.6 HISTOCHEMICAL EVALUATION

4.6.1 IMMUNOFLOURESCENCE

Spinal cord sections (40 μ m, free-floating) were incubated at 4°C for 48 h, whereas sciatic nerve sections (20 μ m), mounted on slides, were incubated at 4°C overnight with the following primary antibodies diluted in PBS-0.3% Triton X-100: 1/200 rabbit polyclonal anti-PK2 (AbCam, Cambridge, UK), 1/200 rabbit polyclonal anti-PKR1 and PKR2 (Alomone Labs, Jerusalem, Israel), 1/500 mouse monoclonal anti-neuronal nuclei (NeuN), 1/400 mouse polyclonal antiglial fibrillary acidic protein (GFAP) (Immunological Sciences, Rome, Italy), 1/300 mouse polyclonal anti-Synaptophysin (Sigma-Aldrich, Milan, Italy), 1/100 rat monoclonal anti-CD11 (BD Pharmigen, Milan, Italy). The sections were then incubated for 2 hours at room temperature in 1:200 anti-species IgG antibodies coupled to Alexa Fluor®-488 or 555 (Immunological Sciences). Nuclei were stained with DAPI 1/500. The stained sections were examined at confocal laser scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany). Immunofluorescence intensity or immunoreactive area was measured in five fields (300 μ m²) for every section in at least 10 sections for every experimental group (<http://imagej.nih.gov/ij/index.html>, free software).

To assess the specificity of the anti-PK2 antibody, we pre-adsorbed it with the protein PK2 (500 ng) overnight at 4°C prior to incubation with tissue. To assess the specificity of the anti-PKR1 and anti-PKR2 antibody, we pre-adsorbed them with the respective blocking peptides (Alomone Labs) overnight.

4.6.2 SCIATIC NERVE IMMUNOHISTOCHEMISTRY

Paraffin-embedded sciatic nerve sections (5 μm), deparaffinized and rehydrated, were incubated with normal horse serum (3%, 1 h, 37°C) then with goat polyclonal anti-PK2 (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, overnight, 4°C), washed and incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) and avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories), stained with 3,3N-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich).

All slides were counterstained with Mayer's haematoxylin, visualized and photographed with an Olympus DP12 microscope equipped with a digital camera. PK2-staining intensity was computed as integrated optical density (IOD) for arbitrary areas and measured in six samples for each experimental group (Image Pro-Plus, 4.5.1, Milan, Italy).

All the experiments for PK2, PKR1 and PKR2 localization in spinal cord and sciatic nerve were conducted in the laboratory of Prof. Lucia Negri (Department of Physiology and Pharmacology "Vittorio Erspamer", University of Rome, La Sapienza).

4.7 DATA ANALYSIS

Results are presented as means \pm SEM. Statistical analyses were performed using one-way or two-way ANOVA for parametric results. Follow-up analysis was performed using the Tukey's test or Bonferroni's post tests for multiple comparisons, respectively. T Student test was used for the comparison between two groups. In the case of non-parametric results, Kruskal-Wallis ANOVA was applied, followed by Dunn's test.

All the statistical analysis was performed using GraphPad Prism 5 Software (San Diego, CA, U.S.A) Differences were considered significant at $p < 0.05$.

5. RESULTS

In order to understand if PK2, PKR1 and PKR2 activities were necessary for the onset, development and resolution of experimental neuropathic pain, we performed *in vivo* and *ex-vivo* studies blocking PKR signalling with the antagonist PC1. PKs system was characterized in two different models of painful neuropathy: a mononeuropathy induced by the chronic constriction injury (CCI) of sciatic nerve and a diabetic polyneuropathy induced by the injection of a pancreatic β cell toxin, streptozotocin (STZ).

The major findings of this study are that (i) PK2 acts as a pro-nociceptive/pro-inflammatory mediator in the pathological cross-talk between neuronal and immune cell in neuropathic pain, (ii) painful manifestations were close related to the up-regulation of PK2 and PKRs in the main anatomical stations of pain transmission, i.e. in spinal cord and in sciatic nerve, (iii) repeated administrations of PC1 efficaciously contrasted neuropathic pain in CCI nerve injured and diabetic mice without producing tolerance. Independently from the etiology, the antagonist PC1 counteracted neuropathy-induced pain hypersensitivity blocking PK2 up-regulation in spinal cord and in the peripheral nervous system and reducing the neuro-inflammatory component related to neuropathic pain development. In addition, (iv) the anti-inflammatory properties of PC1 also ameliorated the course of diabetic neuropathy contrasting the strong inflammatory status associated to the presence of diabetes suggesting a potential role of PK2 also as “modifier” of the pathology.

5.1 PERIPHERAL NERVE INJURY (CCI) MODEL

5.1.1 Effect of PKR blocking on CCI-induced thermal hyperalgesia and mechanical allodynia

Before surgery, baseline thresholds to noxious heat (paw withdrawal latency, PWL) and mechanical stimuli (paw withdrawal threshold, PWT) were similar in all animal groups as well as for the right and left paw.

Chronic constriction injury (CCI) of sciatic nerve induced in animals a precocious establishment of neuropathic pain syndrome. As depicted in figure 1, a marked reduction of the ipsilateral PWL and PWT appeared within few days after surgery reaching the maximal values on day 3 after CCI. Thermal and mechanical sensitivity of the contra-lateral paw remained unchanged, i.e. comparable to the basal level of non-neuropathic animals, for the entire period of the study (data not shown).

On day 3 after surgery, time point corresponding to full development of thermal hyperalgesia and mechanical allodynia, a group of CCI mice was subcutaneously injected with PC1 (150 $\mu\text{g}/\text{kg}$, twice-daily) or saline for 7 days. As controls, a group of sham mice was treated with either PC1 or saline. PC1 dose was chosen as it was the

most effective in order to abolish complete Freund's adjuvant (CFA)-induced thermal hyperalgesia (Giannini et al., 2009).

Repeated administrations of PC1 from day 3 to 9 efficaciously contrasted neuropathic pain. The efficacy of the antagonist to counteract the CCI-induced thermal hyperalgesia and mechanical allodynia was already noticeable 4 days after treatment initiation and was even more evident 3 days later.

Indeed, 7 days of treatment with the antagonist produced a total recovery in the decrease of PWL completely reverting thermal hyperalgesia (panel A). Mechanical allodynia was also significantly reduced in CCI/PC1 treated mice but not abolished. In fact at the end of treatment, the PWT of CCI/PC1 treated mice were significantly higher than the pain thresholds of CCI/saline animals, but not yet comparable to those of controls (panel B).

No differences on the PWL and PWT of sham animals treated with PC1 were observed throughout the experiment.

As thermal and mechanical sensitivity of sham animals were not affected by PC1 administrations and considering the recent European guidelines regulating animal research which prompt to minimize the number of animals, this group of study was not repeated for the successive biochemical evaluations.

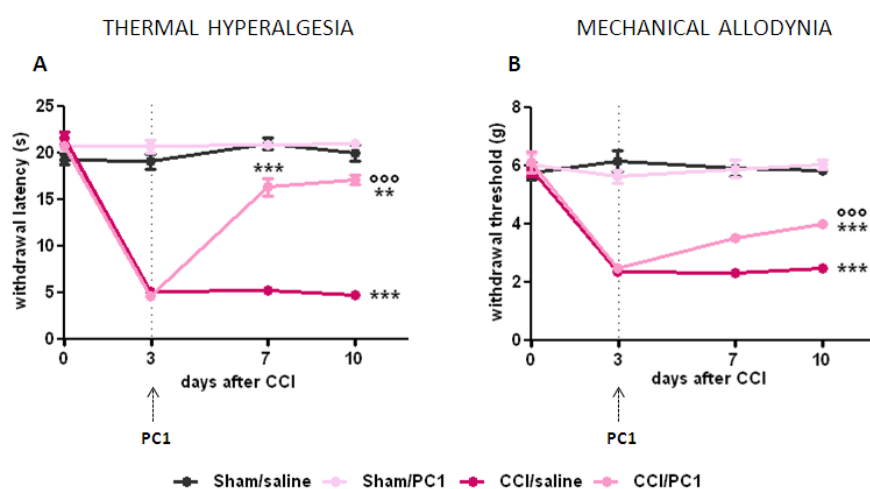


Figure 1. Effect of PC1 (150 µg/kg, twice-daily) s.c. administered to CCI-operated animals for 7 days, starting from 3 days after surgery, on thermal hyperalgesia (A) and mechanical allodynia (B). PWL and PWT were measured by using Plantar Test and Von Frey Test, respectively. Data represent mean ± SEM of 8-10 mice per group. Two way ANOVA was used for statistical evaluation, followed by Bonferroni's test. **p<0.01, ***p<0.001 vs sham/saline; °°°p<0.001 vs CCI/saline.

5.1.2 Prokineticin system localization and CCI-induced modulation

The fact that repeated administrations of PC1 were effective in alleviating painful symptoms clearly indicated the involvement of the PKs system in the development of neuropathic pain. In order to compare the expression of the PKs system in PC1- and saline-treated CCI mice with sham animals injected with only saline, on day 10 after CCI surgery, when the anti-hyperalgesic and anti-allodynic effect of PC1 were greatest, we performed Real Time PCR analysis to evaluate the mRNA levels of PK2, PKR1 and PKR2 in spinal cord and in the sciatic nerve. In the same tissues, immunocytochemistry experiments were conducted to identify the cellular localization of PK2 and its receptors PKR1 and PKR2.

○ PK2, PKR1 and PKR2: mRNA expression and localization in spinal cord

As shown in figure 2, 10 days after surgery, the mRNA levels of PK2 (panel A) and PKR2 (panel C) were markedly higher in L4-L6 spinal cord of CCI-operated mice than sham, whereas no significant difference of PKR1 expression (panel B) was evident in the three groups of study. Repeated PC1 administrations didn't affect PKR1 and PKR2 mRNA levels but succeeded in decreasing the up-regulation of PK2 induced by nerve ligation.

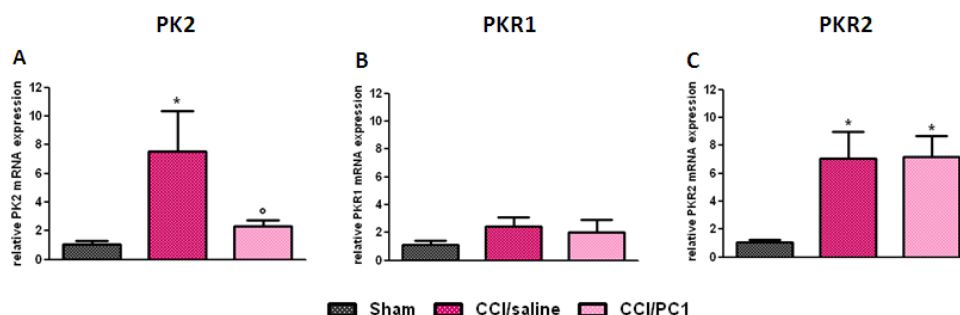


Figure 2. PK2 (A), PKR1 (B), PKR2 (C) mRNA expression in spinal cord (L4-L6) 10 days after CCI surgery, at the end of therapeutic PC1 treatment (s.c. 150 µg/kg, twice-daily for 7 days). The mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in sham animals. Data represent mean ± SEM of 4-6 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$ vs sham/saline; ° $p < 0.05$ vs CCI/saline.

Figure 3 reports the results obtained with PK2 immunofluorescence analysis. PK2 immunofluorescence, localized in superficial layers (I and II) of the spinal cord in sham animals, strongly increased in the ipsilateral dorsal horn 10 days after CCI, staining also the deeper layers. Therapeutic PC1 treatment from day 3 to 9 prevented this increase in immunofluorescence. Indeed, the PK2 signal in CCI/PC1 mice resembled that observed in sham animals (fig.3A, panels a, d and g) mirroring the results of PK2 mRNA evaluation (fig.2, panel A). In spinal cord, sensory primary neurons and astrocytes

appeared to be the main cells involved in CCI-induced PKs system activation. Diffuse punctuate pattern PK2 immunoreactivity partially colocalized with synaptophysin, a presynaptic marker, mainly in superficial laminae of dorsal horn (fig.3B). In CCI/saline animals ipsilateral dorsal horn PK2/GFAP immunoreactivity also increased compared to sham (fig.3A, panel f), indicating that PK2 is associated to GFAP-positive proliferating and activated astrocytes.

As illustrated in figure 3A, panel g, we observed a lower PK2 immunofluorescence induced by 7 days of PC1 treatment in the ipsilateral dorsal horn of CCI/PC1 mice in respect to that of CCI/saline mice (fig.3A, panel d). Moreover, PK2 immunoreactivity too was strongly reduced in the activated astrocytes (fig.3A, panels f and i).

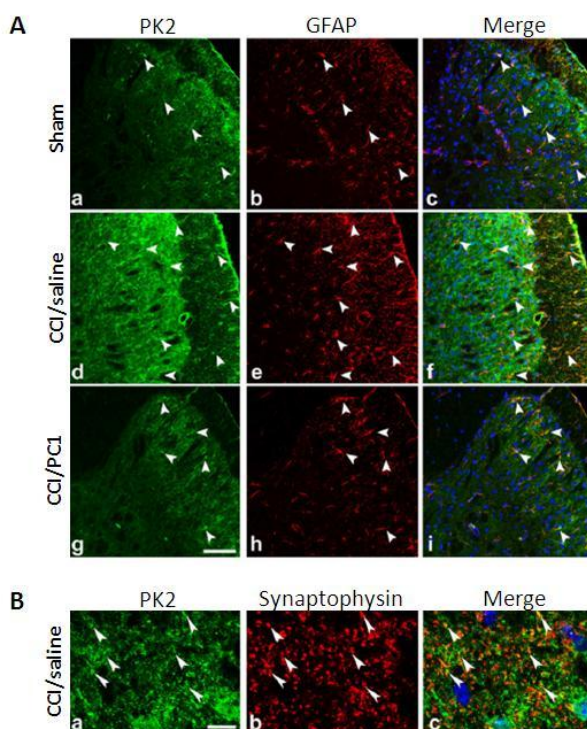


Figure 3. Representative images showing PK2 localization in the mouse L4–L5 spinal cord dorsal horn. **(A)** PK2-positive profiles (green) in sham (a), CCI/saline (d) and CCI/PC1 (g) mice. GFAP (astrocyte marker) positive profiles (red) in sham (b), CCI/saline (e) and CCI/PC1 (h) mice. Sciatic nerve ligation induced a substantial increase in PK2 and in GFAP signal 10 days after ligation. Double staining reveals a colocalization of PK2 with the astrocyte marker GFAP (f). Scale bar: 50 μm. **(B)** Representative images showing colocalization (c) of PK2 (green, a) with synaptophysin (red, b). Scale bar: 10 μm. Cell nuclei were counterstained with DAPI (blue).

Spinal cord localization of PKR1 and PKR2 is depicted in figure 4. The PKR1 signal in the spinal cord sections was very faint and was unaffected by CCI or by PC1 treatment. This receptor clearly colocalized only with GFAP-positive astrocytes (fig.4, panels D-F) whereas we never detected PKR1 signals in NeuN (neuronal marker)-positive cells (fig.4, panel H).

In sham animals, PKR2 signal was clearly evident in superficial laminae of the dorsal horn, localized with positive neuronal cell bodies (fig.4, panel A, arrows and insert) and

some astrocytes (fig.4, panel A, arrowheads). 10 days after CCI, PKR2 positive neuronal cell bodies were more evident also in deeper layers of the dorsal horn (panel B, arrows and insert) as illustrated in the panel 4 G, showing the colocalization of PKR2 with the NeuN positive cells. As PK2, the PKR2 signal clearly increased after nerve ligation in the activated astrocytes (panel B, arrowheads and insert) and also in the diffuse punctuate pattern. Repeated PC1 administrations didn't induce any variations in PKR2 immunoreactivity (panel C).

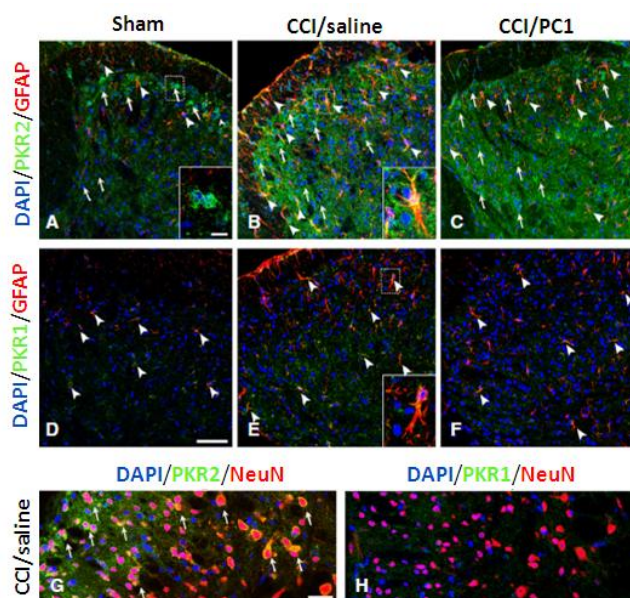


Figure 4. Representative images showing PKR2 and PKR1 localization in the mouse L4–L5 spinal cord dorsal horns from 10 days-sham (**A**, **D**), CCI/saline (**B**, **E**) and CCI/PC1 (**C**, **F**) mice. PKR2 immunofluorescence (green) is clearly evident in sham animals, localized in some neuronal cells (**A**, arrows and insert), and in some astrocytes (**A**, arrowheads). 10 days after CCI (**B**), PKR2 positive neuronal cell bodies were more evident also in deeper layers of the dorsal horn (**B**, arrow) as demonstrated by colocalization with the neuronal marker NeuN (**G**). The localization of PKR2 in activated astrocytes is demonstrated by the double staining of PKR2 (green) with the astrocytes marker GFAP (**B**, arrowheads and insert). The diffuse punctuate pattern PKR2 signal appeared clearly increased. Therapeutic PC1 treatment (s.c. 150 μ g/kg, twice-daily for 7 days) did not modify the PKR2 immunofluorescence intensity. In the spinal cord, the PKR1 signal was very faint and was not affected by nerve injury nor by PC1 treatment. PKR1 immunoreactivity was clearly evident in GFAP-positive resting and activated astrocytes (**D**, **E**) and was not modified by PC1 treatment. We never detected PKR1 signal in NeuN-positive cells. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 50 μ m in A to F; 30 μ m in G, H and 10 μ m inserts.

○ *PK2, PKR1 and PKR2: mRNA expression and localization in sciatic nerve*

Ten days after surgery, a general activation of the PKs system took place in the sciatic nerve (fig.5). In CCI mice the mRNA levels of PK2 (panel A), PKR1 (panel B) and PKR2 (panel C) were significantly increased in respect to the basal levels of the respective controls. PKR2 up-regulation appeared particularly evident, since the receptor expression was about 150 folds higher than in sham mice. Repeated administrations of PC1 from day 3 to 9 markedly reduced the overexpression of PK2, without affecting the expression levels of the receptors.

Immunohistochemical staining using anti-PK2 antibody failed to demonstrate any PK2 immunoreactivity in the sciatic nerve of sham mice whereas, 10 days after CCI, a heavy infiltration of PK2-positive cells (brown colour) was evident in the neuroma in the immediate proximity of the injury, as illustrated in figure 6 (panel A). Systemic PC1 administrations significantly reduced PK2 immunoreactivity in the cytoplasm of these cells (panel A, insert) as demonstrated in panel B by quantitative analysis of PK2 signal computed as integrated optical density (IOD), reflecting the lower levels of PK2 mRNA measured in the injured nerve of CCI/PC1 mice at the end of 7 days of treatment (fig.5, panel A). Immunofluorescence staining of the neuroma in CCI/saline mice demonstrated that PK2 signal was associated with GFAP-positive Schwann cells (panel C) and with CD11b-positive neutrophils and macrophages (panel D). PKR1 (panel E) was mainly associated with CD11b-positive cells, suggesting that the PKR1 mRNA increase depends on the high number of infiltrating cells (Giannini et al., 2009), whereas PKR2 was mainly associated with GFAP-positive cells (panel F). As shown in figure 7, panels B and E, a dramatic increase of PK2 and PKR2 immunofluorescence was observed in nerve fibres and GFAP-positive structures 10 days after nerve ligation. Accordingly with the PK2- and PKR2- mRNA analysis, in CCI/PC1 mice 7 days of treatment with the PKR antagonist strongly reduced PK2 signal (panel C), but was ineffective against PKR2 up-regulation (panel F).

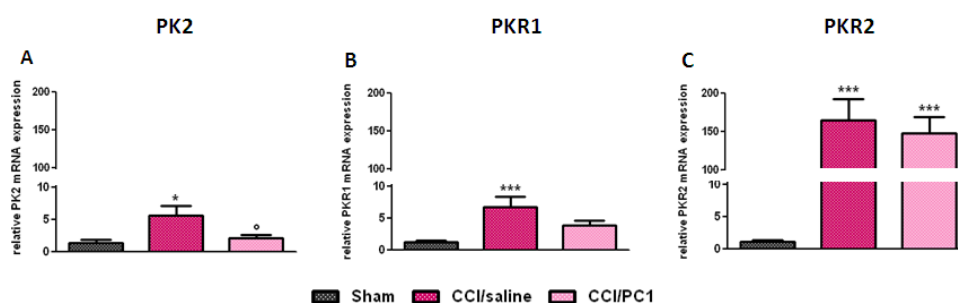


Figure 5. PK2 (A), PKR1 (B), PKR2 (C) mRNA expression in ipsilateral sciatic nerve 10 days after CCI surgery, at the end of therapeutic PC1 treatment (s.c. 150 µg/kg, twice-daily for 7 days). The mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in sham animals. Data represent mean ± SEM of 8-10 samples per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. *p<0.05, ***p<0.001 vs sham/saline; °p<0.05 vs CCI/saline.

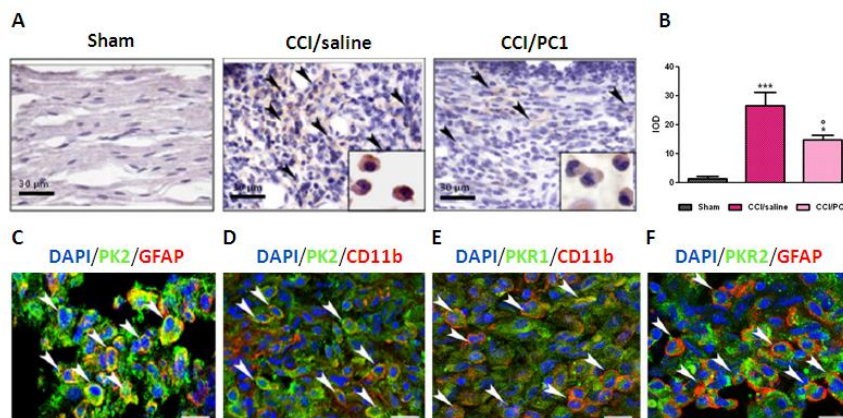


Figure 6. Representative images of sciatic nerve in the immediate proximity of the injury. **(A)** Immunohistochemical staining of ipsilateral sciatic nerve, on day 10 after CCI, from sham, CCI/saline and CCI/PC1 mice with anti-PK2 antibody and haematoxylin. Scale bar = 30 μ m. Arrowheads indicate the infiltrating cells expressing the PK2 protein. A sustained infiltration of PK2-positive cells was evident 10 days after CCI. Therapeutic PC1 treatment (s.c. 150 μ g/kg, twice-daily for 7 days) significantly reduced the PK2 immunoreactivity (brown colour) in the cytoplasm of these cells (insert) as demonstrated in **(B)** by quantitative analysis of PK2 signal computed as integrated optical density for arbitrary areas (six sections per animal, six animals). Data are means \pm SEM of 4-6 animals. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons * p <0.05, *** p <0.001 vs sham; * p <0.05 CCI/saline.

(C and D) Immunofluorescence double staining showing colocalization (yellow, arrowheads) of PK2 (green) with GFAP (Schwann cell marker, red) and CD11b (macrophage marker, red) in the immediate proximity of the injury in the sciatic nerve of CCI/saline mice. **(E and F)** Representative images showing the localization (arrowheads) of the receptor PKR1 (green) in CD11b-positive macrophages (red) and of the receptor PKR2 (green) in GFAP-positive Schwann cells (red) in the immediate proximity of the injury of the sciatic nerve in CCI/saline mice. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 20 μ m.

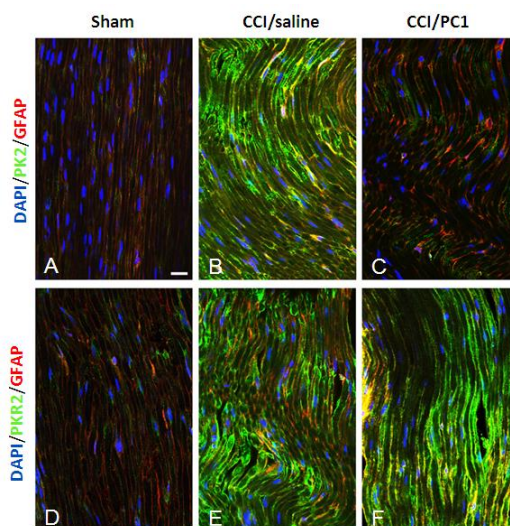


Figure 7. Representative images of CCI-induced up-regulation of PK2 and PKR2 in the longitudinally sliced sciatic nerve proximal to the lesion. PK2 immunofluorescence was never found in uninjured nerve **(A)**. Only a very faint PKR2 signal was evident in the non-activated Schwann cells (GFAP-positive cells, red) **(D)**. A dramatic increase of PK2 and PKR2 signal **(B and E, green)** in fibres and in GFAP-positive structures was evident 10 days after nerve ligation. PC1 treatment prevented the injury-induced PK2 up-regulation **(C)** but was ineffective against PKR2 up-regulation **(F)**. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar: 20 μ m.

5.1.3 Effect of therapeutic PKR blocking on cytokines levels in spinal cord and sciatic nerve

It is well known that pro- and anti-inflammatory cytokines play an important role in CCI-induced neuropathic pain (Martucci et al., 2008). We therefore evaluated the ability of PC1 treatment to modulate neuroinflammation in this model. As cytokines are regulated at several post-transcriptional and post-translational levels, we measured both mRNA and protein levels. As illustrated in figures 8 and 9, 10 days after CCI surgery, mRNA and protein levels of IL-1 β were increased in spinal cord (fig.8, panels A and B) and in the injured sciatic nerve (fig.9, panels A and B). Repeated PC1 administrations efficaciously contrasted the IL-1 β increase, restoring cytokine levels to basal values in the spinal cord (fig.8, panels A and B) and significantly reducing them in the sciatic nerve (fig.9, panels A and B).

In our experimental setting, we didn't find any modulation of IL-10 in the spinal cord, neither after CCI nor PC1 treatment (fig.8, panels C and D). On the contrary, a clear alteration of IL-10 expression pattern was observed in the sciatic nerve. Ten days after surgery, the concentration of IL-10 protein decreased in injured nerve (fig.9, panel C), whereas its mRNA expression increased (fig.9, panel D). These modifications are probably due to the activation of the synthetic machinery of IL-10 in order to counteract the proinflammatory cascade induced by the lesion (Sacerdote et al., 2013). PC1 treatment was effective in augmenting the level of IL-10 protein, which was significantly reduced in the sciatic nerve of CCI/saline mice (fig.9, panel C).

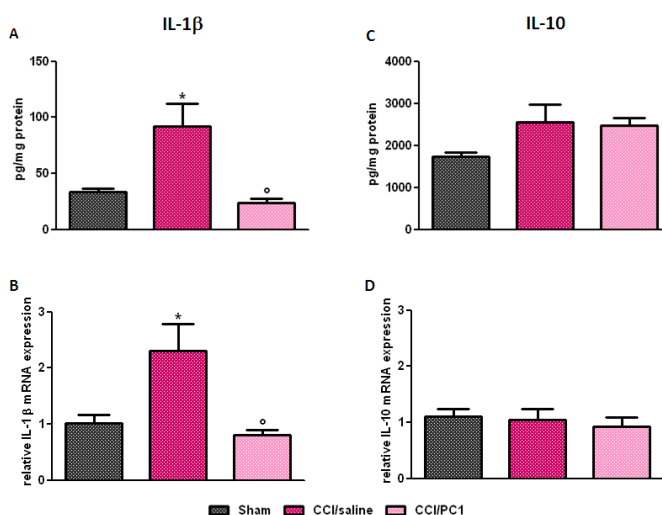


Figure 8. Effect of PC1 on IL-1 β and IL-10 protein content (A and C) and mRNA expression (B and D) in spinal cord 10 days after CCI surgery, at the end of therapeutic PC1 treatment (s.c. 150 μ g/kg, twice-daily for 7 days). Cytokine protein content, evaluated by ELISA, was reported as pg/mg of protein. Cytokine mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in sham animals. Data represent mean \pm SEM of 4-6 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. *p<0.05 vs sham/saline; °p<0.05 vs CCI/saline.

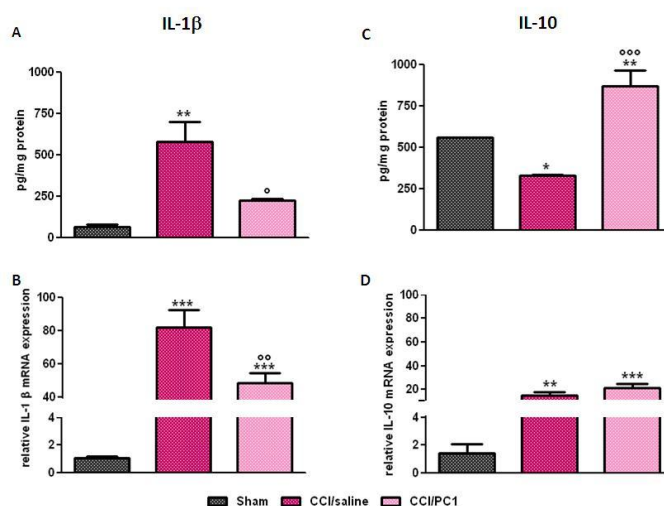


Figure 9. Effect of PC1 on IL-1 β and IL-10 protein content (A and C) and mRNA expression (B and D) in ipsilateral sciatic nerve 10 days after CCI surgery, at the end of therapeutic PC1 treatment (s.c. 150 μ g/kg, twice-daily for 7 days). Cytokine protein content, evaluated by ELISA, was reported as pg/mg of protein. Cytokine mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in sham animals. Data represent mean \pm SEM of 4-6 samples per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs sham/saline; ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$ vs CCI/saline.

5.2 DIABETES-INDUCED NEUROPATHIC PAIN MODEL

Numerous conditions leading to neuropathic pain syndrome have been identified in human. Among these, one of the most frequent cause is the presence of diabetes. Nowadays, diabetes is considered to be a challenging health problem as it affects million people in the world and its prevalence has been projected to increase twofold in the next twenty years.

Since diabetes incidence is rapidly growing, the identification of new molecular targets for treating neuropathic pain is of central importance. In this direction and supported by the results obtained from CCI model, we have considered of great interest to investigate the role of the PKs system also in neuropathic pain induced by diabetes.

5.2.1 Effect of therapeutic PKR blocking on hyperglycaemia, mechanical allodynia and body weight loss in experimental diabetic model induced by the administration of a single high dose or repeated multi-lower doses of STZ: similarities and differences.

In order to study whether the blocking of PKR was effective in alleviating diabetic neuropathic pain we have performed a series of experiments using a mouse model of diabetes induced by chemical destruction of pancreatic insulin-secreting β -cells. We

first tested the effect of PC1 in mice injected with a single high dose of β -cell toxin, streptozotocin (STZ, 200 mg/kg).

A single STZ administration induced in animals a rapid establishment of hyperglycaemic state associated to the development of painful symptoms. As depicted in figure 10 (panel A), blood glucose levels of STZ mice strongly increased after 7 days from diabetes induction and were still significantly higher on day 35 compared to normo-glycaemic values of controls. A marked reduction of the PWT of mice appeared within few days after diabetes induction reaching the maximal values on day 14 after STZ injection (fig.10, panel B). Pain thresholds to mechanical stimuli of STZ mice remained significantly lower than that of the controls, i.e. non-diabetic mice, for the entire period of observation.

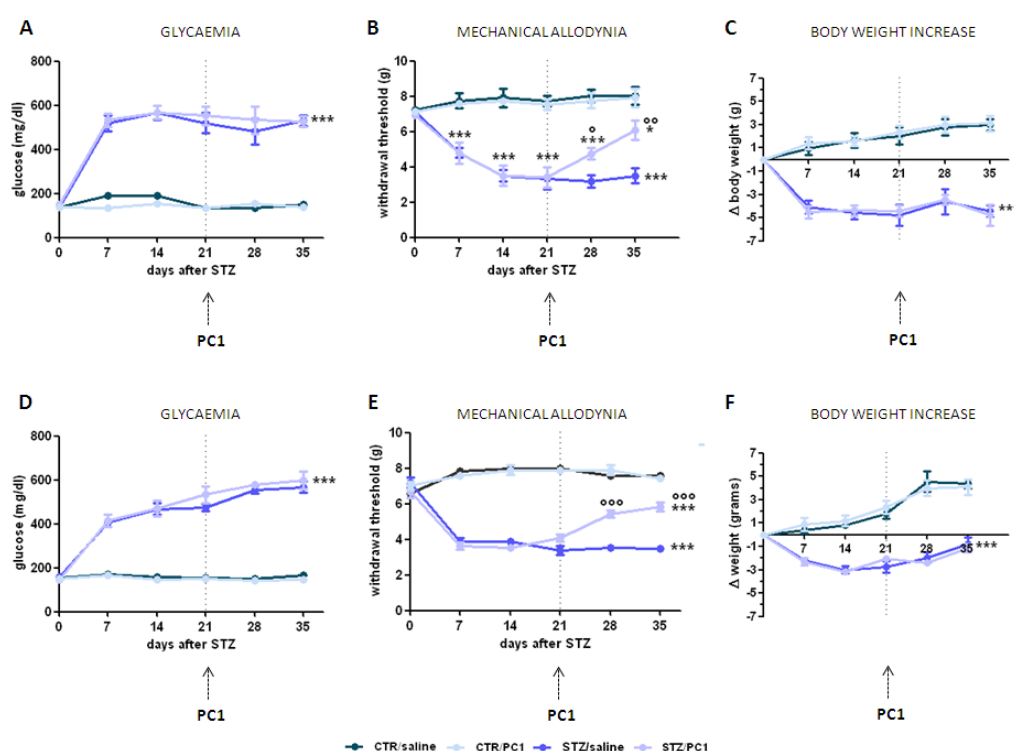


Figure 10. Effect of PC1 (150 μ g/kg, twice-daily) s.c. administered to mice injected with a single high dose of streptozotocin (STZ, 200 mg/kg) (A-C) or with multiple low-dose of streptozotocin (MLD-STZ, 80 mg/kg for 3 consecutive days) (D-F) for 14 days, starting from 21 days after diabetes induction (therapeutic treatment), on glycaemic values (A, D), mechanical allodynia (B, E) and body weight increase (C, F). Data represent mean \pm SEM of 4-6 mice per group. Two way ANOVA was used for statistical evaluation, followed by Bonferroni's test. * p <0.05, *** p <0.001 vs CTR/saline; ° p <0.05, °° p <0.01, °°° p <0.001 vs STZ/saline.

Allodynia is known to be a characteristic symptom of the diabetic painful neuropathy. On the contrary, the sensitivity of animals to thermal stimuli changes during the progression of the diabetes. Indeed, in the early stage of the disease mice exhibit thermal hypersensitivity, while advanced stage of diabetes is typically characterized by

thermal hypoalgesia caused by sensory loss (Ulugol et al., 2012). For this reason, we decided to evaluate only the sensitivity to mechanical stimuli.

The antagonist PC1 was s.c. administered to STZ animals twice-daily for 14 days, starting on day 21 after diabetes induction when neuropathic pain and hyperglycaemia were fully developed. PC1 was given to animals at the dose of 150 µg/kg, the same dosage used in order to counteract CCI-induced pain hypersensitivity (see figure 1). As controls, a group of STZ mice treated with saline and non-diabetic mice treated with either PC1 or saline were used.

Repeated administrations of PC1 from day 21 to 34 didn't affect blood glucose levels (fig.10, panel A). Glucose concentration of non-diabetic control mice treated either PC1 or saline remained unchanged and was normal throughout the study. However, blocking PKRs contrasted neuropathic pain. Therapeutic PC1 treatment significantly reduced mechanical allodynia in STZ mice without abolishing it. As shown in panel B (fig.10), 14 days after treatment initiation, the PWT of STZ/PC1 treated mice were significantly higher than PWT of STZ mice treated with only saline, but not yet comparable to those of controls. PWT of non-diabetic animals treated with PC1 remained unchanged and similar to basal thresholds of controls throughout the experiment.

Unfortunately, high dose of STZ dramatically compromised the health status of the animals. As illustrated in panel C (fig.10), STZ administration rapidly produced in animals a marked reduction of the body weight. Moreover, an exacerbation of other classical symptoms associated to chronic hyperglycaemia, including blindness, polyuria, polydipsia, loss of appetite, was evident leading us to doubt about the reliability of the results.

As the final aim of this study was the characterization of new target for the development of an alternative strategy for contrasting neuropathic pain in human we have planned a new series of experiments replacing the single high dose of STZ-induced diabetes model, which is ultimately considered to be inappropriate for resembling the clinical manifestations of diabetic neuropathy, with that induced by multi-lower doses of streptozotocin (MLD-STZ, 80 mg/kg).

In this model blood glucose levels gradually increased after STZ injections reaching the maximal values 35 days after diabetes induction and, similarly to what observed in the first model used, hyperglycaemia was already present 7 days after STZ injections, as shown in panel D (fig.10). In parallel, a consistent development of mechanical allodynia occurred within few days after diabetes induction (fig.10, panel E). On day 14 after MLD-STZ injection the PWT of diabetic mice were markedly reduced than that of the controls and remained significantly lower for the entire period of the study.

As shown in panels D and E, therapeutic PC1 administration from day 21 to 34 was not able to modify blood glucose levels of either MLD-STZ or control mice, while it was effective in contrasting neuropathic pain, significantly enhancing the PWT of MLD-STZ

mice after chronic treatment in respect to diabetic mice administered with saline only. PWT of control animals were never affected by chronic treatment with PC1, remaining similar to basal thresholds of saline treated-controls mice throughout the experiment. MLD-STZ administrations produced in mice the classical features of diabetes without drastically compromising their general well-being status. In respect to non-diabetic control animals, a significant body weight loss was still observed in diabetic mice (panel F). However, this loss was milder in comparison to the effect induced by a single high STZ dose (panel C).

Therapeutic PC1 treatment did not influence body weight in mice injected with either a single high dose of STZ or MLD-STZ, or in the respective non-diabetic controls, as illustrated in panels C and F.

MLD-STZ model was used in all subsequent experiments and, as explained for the CCI model (see chapter 5.1.1), the group of control mice treated with the antagonist (CTR/PC1 group) was excluded from the successive studies.

5.2.2 Prokineticin system and diabetic neuropathic pain-modulation

- *Parallelism between PKs system activation and development of diabetes induced-mechanical allodynia.*

Mechanical allodynia induced by MLD-STZ was clearly evident 7 days after diabetes induction. It reached full development on day 14 and was still significant on day 56 compared to controls, as illustrated in figure 11, panel A.

A single bolus systemic injection of PC1 (150 µg/kg) on day 21 after diabetes induction, when the neuropathic pain syndrome was overt, produced a total recovery in the decreased PWT of MLD-STZ mice abolishing the established mechanical allodynia in 30 minutes. The anti-allodynic effect lasted for about 2 hours and gradually disappeared within 4 hours after the PC1 administration (fig.11, panel B).

As previously described (fig.10, panel E), therapeutic PC1 administration from day 21 to 34 after diabetes induction was effective in alleviating diabetes-induced mechanical allodynia. Interestingly, this treatment schedule delayed painful symptom reappearance after PC1 suspension, leading us to suppose that blocking PK2 signalling could induce permanent changes in neuronal circuits involved in the maintenance of neuropathic pain. As shown in panel A in fact, at the end of the chronic treatment with PC1, the PWT of MLD-STZ mice were markedly higher in respect to the PWT of diabetic mice treated with only saline; after treatment discontinuation the PWT remained significantly elevated although a progressive decrease of the thresholds started to be evident.

In order to correlate the activation of the PKs system with the development of diabetic neuropathic pain we performed Real-Time PCR analysis for determining PK2 mRNA

expression levels in the spinal cord of MLD-STZ mice at different time from diabetes induction. The time-course of PK2 mRNA expression is depicted in figure 11, panel C. A noticeable increase of PK2 mRNA expression appeared in the spinal cord of diabetic mice 7 days after the first STZ administration, time point corresponding to clear manifestation of painful symptoms. The augmentation of PK2 mRNA level was even more evident 7 days later and was still significant on day 56, demonstrating a close parallelism between the changes in pain behaviour and the PKs system activation (panels A and C).

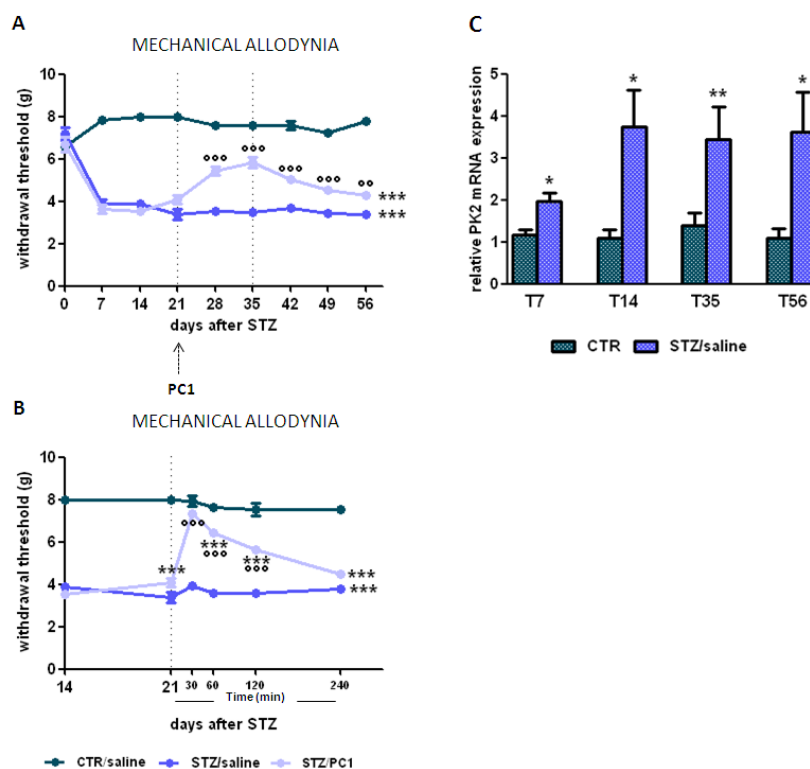


Figure 11. Long-lasting effect of therapeutic PC1 administrations (s.c. 150 $\mu\text{g}/\text{kg}$, twice-daily for 14 days, from day 21 to 34 after diabetes induction) in mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) on mechanical allodynia (A). Anti-allodynic effect of PC1 administration as a single bolus (B). Data represent mean \pm SEM of 4 mice per group. Two way ANOVA was used for statistical evaluation, followed by Bonferroni's test. *** $p < 0.001$ vs CTR/saline; ** $p < 0.01$, *** $p < 0.001$ vs STZ/saline. Time-course of mRNA expression levels of PK2 in spinal cord (L4-L6) of mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) at different times after diabetes induction (C). The mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in CTR animals. Data represent mean \pm SEM of 4-6 mice per group. Student T Test was used for statistical evaluation * $p < 0.05$, ** $p < 0.01$ vs CTR.

- *Effect of therapeutic PKR blocking on PK2-, PKR1- and PKR2-mRNA expression in spinal cord and sciatic nerve*

At the end of therapeutic PC1 administrations, when the anti-allodynic effect of the antagonist was maximal (i.e. 35 days after STZ), we performed Real Time PCR analysis in diabetic neuropathic mice treated with either PC1 or saline and in non-diabetic

control animals to compare mRNA levels of PK2, PKR1 and PKR2 at the main central and peripheral sites of pain transmission, i.e. in spinal cord and in the sciatic nerve. As shown in figure 12, panel A (a-c), a clear activation of the PKs system took place in the spinal cord of diabetic mice 35 days after MLD-STZ injections. The mRNA levels of PK2 (a) as well as PKR2 (c) were markedly higher in L4-L6 spinal cord of MLD-STZ injected mice than values measured in non-diabetic controls, whereas no significant difference of PKR1 expression (b) was evident in the three groups of study. A reduction of PK2 and PKR2 up-regulation was evident in MLD-STZ animals administered with PC1 compared to diabetic mice treated with saline. Indeed PK2 and PKR2 up-regulation was no more statistically significant in comparison to normal animals, although PK2 and PKR2 expression were not yet statistically different from diabetic mice.

We also analysed the mRNA expression levels of PK2 in spinal cord 56 days after diabetes induction, 21 days after PC1 treatment discontinuation, when a significant anti-allodynic effect of the antagonist was still evident in MLD-STZ mice. As illustrated in panel d (fig. 12), in presence of an overt mechanical allodynia, the level of PK2 mRNA expression was higher in spinal cord of MLD-STZ in respect to basal levels of the controls. Chronic PC1 administrations from day 21 to 34 after diabetes, prevented the up-regulation of PK2 in spinal cord of diabetic mice also at later times.

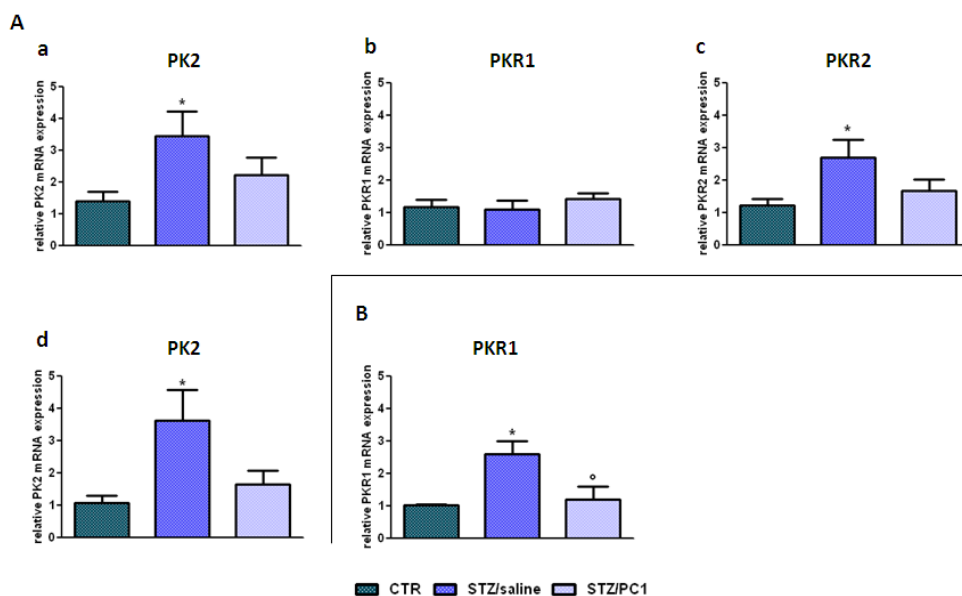


Figure 12. (A) PK2 (a), PKR1 (b), PKR2 (c) mRNA expression in spinal cord (L4-L6) 35 days after diabetes induction with MLD-STZ (80 mg/kg for 3 consecutive days), at the end of therapeutic PC1 treatment (s.c. 150 µg/kg, twice-daily for 14 days) and (d) PK2 mRNA expression in spinal cord (L4-L6) 56 days after diabetes induction with MLD-STZ, 21 days after the discontinuation of the therapeutic PC1 treatment. (B) mRNA expression in sciatic nerve 35 days after diabetes induction with MLD-STZ (80 mg/kg for 3 consecutive days), at the end of therapeutic PC1 treatment (s.c. 150 µg/kg, twice-daily for 14 days). The mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in CTR animals. Data represent mean \pm SEM of 4-6 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$ vs CTR; ° $p < 0.05$ vs STZ/saline.

Thirty-five days after diabetes induction, in the sciatic nerve of MLD-STZ we also observed a significant increase of PKR1 mRNA levels. Repeated PC1 administrations were effective in reducing the neuropathy-induced PKR1 up-regulation, bringing its levels to basal (fig.12, panel B). Real-Time PCR analysis for PK2 and PKR2 mRNA evaluation failed to determine the expression levels of PK2 and PKR2 in the sciatic nerve. Amplification signal for PK2 and PKR2 was detected too late ($C_T > 38$) in samples from MLD-STZ injected mice treated with PC1 and non-diabetic animals, impeding to compare their expression among the three groups of study (data not shown).

- *Effect of preventive PKR blocking on diabetes-induced mechanical allodynia and PK2 up-regulation in spinal cord.*

Data above described clearly demonstrated the involvement of the PKs system in diabetic neuropathic pain leading us to suppose a possible implication of the system not only in the maintenance but also in the onset of painful syndrome. In order to check this hypothesis, we performed a precocious blocking of the PKRs in MLD-STZ mice not yet neuropathic.

A group of animals was s.c. administered with the antagonist PC1 for 14 days, starting on day 0, time point corresponding to the first STZ injection. As controls mice injected with MLD-STZ treated with only saline and non-diabetic mice were used.

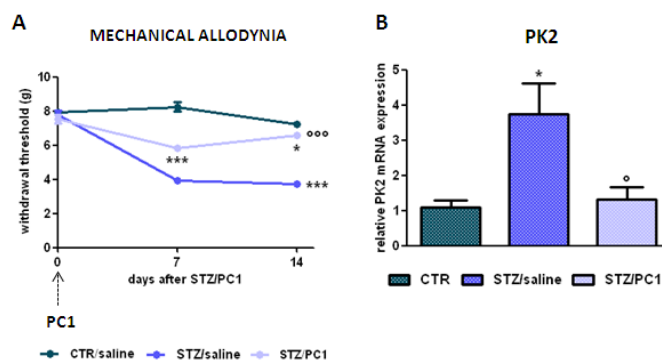


Figure 13. Effect of PC1 (150 μ g/kg, twice-daily) s.c. administered to mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) for 14 days, starting from day 0, time point corresponding to the first STZ administration (preventive treatment), on mechanical allodynia (A). Data represent mean \pm SEM of 5 mice per group. Two way ANOVA was used for statistical evaluation, followed by Bonferroni's test. * $p < 0.05$, *** $p < 0.001$ vs CTR/saline; °°° $p < 0.001$ vs STZ/saline. PK2 mRNA expression in spinal cord (L4-L6) 14 days after the initiation of preventive PC1 treatment/the first MLD-STZ (80 mg/kg for 3 consecutive days) injection (B). The mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in CTR animals. Data represent mean \pm SEM of 6-8 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$ vs CTR; ° $p < 0.05$ vs STZ/saline.

As depicted in figure 13, panel A, early PC1 administrations from day 0 to 13 after diabetes induction significantly counteracted diabetes-induced neuropathic pain, preventing the development of painful symptoms in MLD-STZ mice. On day 7 after diabetes induction, when mechanical allodynia was fully developed in MLD-STZ mice, the PWT of diabetic mice precociously treated with the antagonist PC1 appeared significantly higher respect to MLD-STZ animals and this difference of mechanical sensitivity was even more evident 7 days later. Indeed, at the end of preventive treatment, mechanical nociceptive thresholds of mice treated with chronic PC1 were almost comparable to those evaluated in non-diabetic control mice.

To correlate the changes in pain behaviour with the PKs system activation, we performed Real-Time PCR analysis to compare the mRNA expression level of PK2 in spinal cord of MLD-STZ mice treated either with preventive PC1 administrations or saline, 14 days after the first STZ injection.

Precocious treatment with PC1 efficaciously contrasted neuropathy-induced PK2 up-regulation, significantly preventing the augmentation of PK2 expression, which strongly increased in spinal cord of MLD-STZ mice 14 days after diabetes induction, as reported in panel B.

- *Spinal cord glutamate receptor modulation by diabetes and preventive PC1 treatment*

Glutamate is one of the main mediator in pain processing and it is known to participate in the alteration of the synaptic transmission during neuropathic pain (Iwata et al., 2007; Daulhac et., 2011).

In order to further support the anti-allodynic effect of PC1, we also analyzed the expression of glutamate NMDA receptor subunits N1, N2A and N2B and the glutamate AMPA receptor subunits A1 and A2/3 in spinal cord of MLD-STZ mice treated with preventive PC1 administrations.

As illustrated in figure 14, spinal cord N1, N2A and N2B (panels A-C) and A1 and A2/3 (panels D and E) are shown as grey density percentages of non-diabetic control mice (normalized on tubulin) and in the form of a representative western blot. Subunit content was also normalized on Na/K ATPase, endogenous control for the integrity of plasma membrane proteins. As depicted in panel F, protein levels of both tubulin and Na/K ATPase were comparable in spinal cord of MLD-STZ injected mice treated either with PC1 or saline and non-diabetic animals indicating that tubulin and Na/K ATPase protein content was not altered neither after PC1 administrations nor STZ injections.

Fourteen days after diabetes induction, an alteration of the expression pattern of the subunits N2A and N2B was clearly evident in spinal cord of MLD-STZ mice. As reported in panels B and C, in presence of fully developed mechanical allodynia, a decrease of the NMDA receptor subunit N2A was present, while the expression of the N2B subunit

significantly increased compared to basal levels of controls. Early PC1 administrations from day 0 to 13 after diabetes induction was effective in preventing N2B up-regulation in spinal cord of diabetic mice (panel C), without affecting the levels of the subunit N2A (panel B). In our experimental setting, we didn't find any modulation of the subunit N1, A1 and A2/3 expression in the spinal cord, neither after diabetes induction nor preventive PC1 treatment (panels A, D and E).

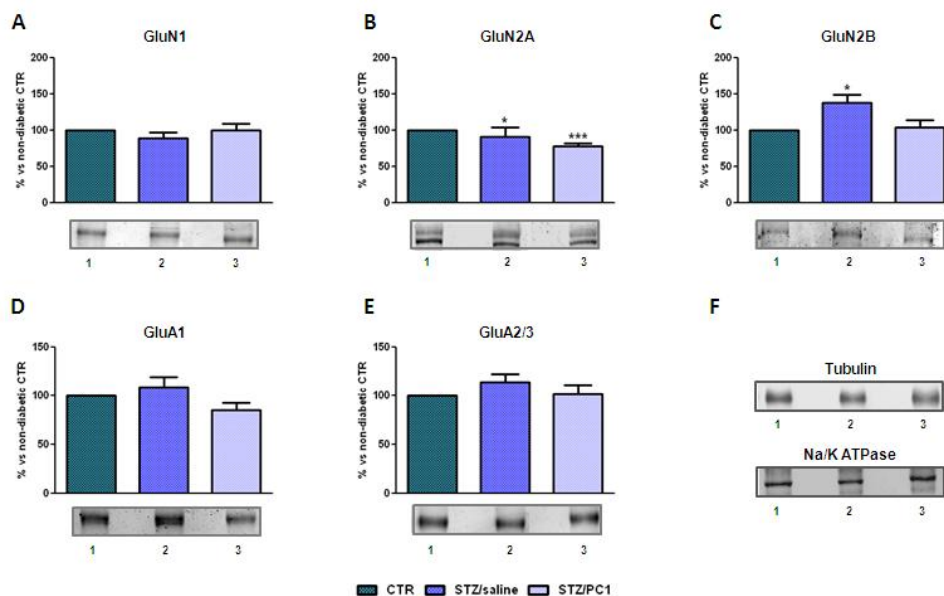


Figure 14. Effect of preventive PC1 administrations (s.c. 150 μ g/kg, twice-daily for 14 days) on GluN1 (A), GluN2A (B), GluN2B (C), GluA1 (D) and GluA2/3 (E) subunit content and representative Western blot bands in spinal cord (L4-L6) of mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) 14 days after diabetes induction, at the end of PC1 treatment. Lanes 1 are extracts of CTR non-diabetic mice, lanes 2 from diabetic STZ/saline mice and lanes 3 from diabetic STZ/PC1 mice. Grey levels of glutamate receptor subunits were normalized on tubulin and expressed as optical density ratio calculated by taking the optical density of the control as 100%. Na/K ATPase was used as controls for the integrity of plasma membrane proteins (F). The values are the mean \pm S.E.M. of 7-8 separate experiments for each antibody. Kruskal-Wallis non parametric ANOVA was used for statistical evaluation, followed by Dunn's test for multiple comparisons. * p <0.05, *** p <0.01 vs CTR.

5.2.3 Effect of therapeutic PKR blocking on cytokines levels in spinal cord and sciatic nerve.

In order to determine the effect of therapeutic PKR blocking on the neuroinflammation associated to diabetic neuropathic pain development we evaluated the expression of IL-1 β and IL-10 in spinal cord and sciatic nerve measuring both cytokine mRNA levels and protein content. As illustrated in figures 15 and 16, 35 days after MLD-STZ injections, mRNA and protein levels of IL-1 β were increased in spinal cord (fig.15, panels A and B) and in the sciatic nerve (fig.16, panels A and B) of diabetic mice. Repeated PC1 administrations efficaciously contrasted IL-1 β up-regulation, restoring cytokine levels to basal values in the spinal cord (fig.15, panels A and B) and significantly reducing them in the sciatic nerve (fig.16, panels A and B).

In spinal cord no modulation of IL-10 protein levels was ever observed, either after diabetes induction or PC1 treatment (Fig.15, panels C). On the contrary, the evaluation of mRNA expression revealed a slight decrease of IL-10 levels in spinal cord from MLD-STZ mice which PC1 administrations completely prevented, as depicted in panel D (fig. 15).

Thirty-five days after diabetes induction, in sciatic nerve of MLD-STZ mice the protein content and the mRNA levels of IL-10 were decreased compared to the basal levels of the controls (fig.16, panels C and D). PC1 administrations contrasted the significant decrease of IL-10 protein, enhancing cytokine production, as illustrated in panel C. Interestingly, PC1 led the mRNA expression of IL-10 above the physiological levels of the controls, shifting the immunity of the nerve towards an anti-inflammatory profile (panel D).

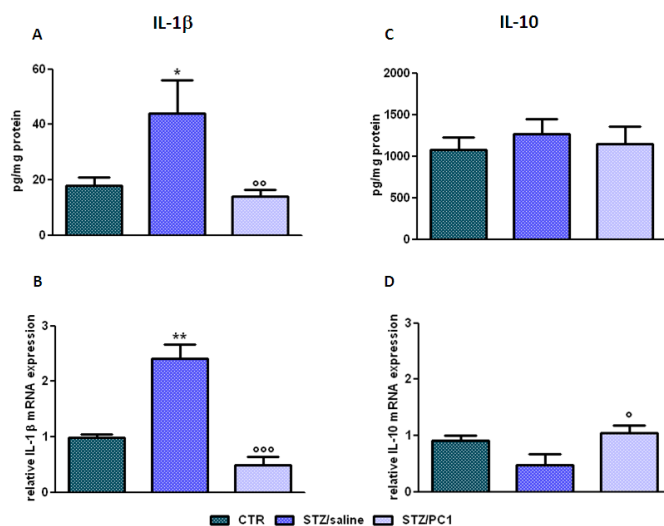


Figure 15. Effect of PC1 administrations on IL-1 β and IL-10 protein content (A and C) and mRNA expression (B and D) in spinal cord 35 days after diabetes induction with MLD-STZ (80 mg/kg for 3 consecutive days) administration, at the end of therapeutic treatment with PC1 (s.c. 150 μ g/kg, twice-daily for 14 days) starting on day 21 from the first STZ injection. Cytokine protein content, evaluated by ELISA, was reported as pg/mg of protein. Cytokine mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in CTR animals. Data represent mean \pm SEM of 6-8 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$ vs CTR; ^o $p < 0.05$, ^{oo} $p < 0.01$, ^{ooo} $p < 0.001$ vs STZ/saline.

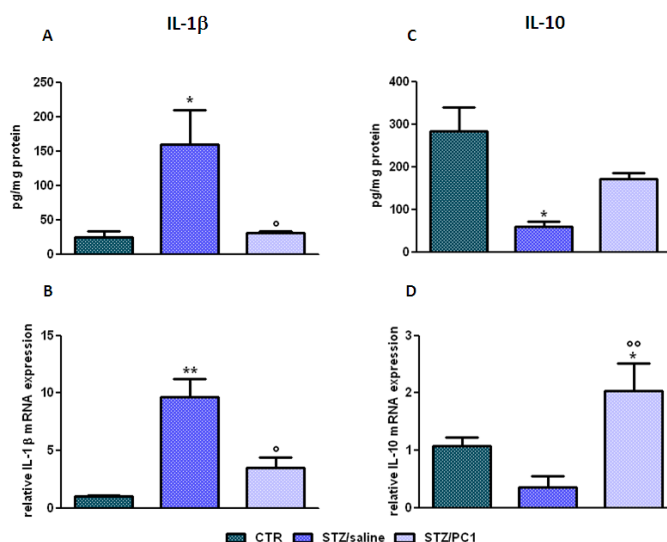


Figure 16. Effect of PC1 administrations on IL-1 β and IL-10 protein content (A and C) and mRNA expression (B and D) in sciatic nerve 35 days after diabetes induction by MLD-STZ (80 mg/kg for 3 consecutive days), at the end of therapeutic treatment with PC1 (s.c. 150 μ g/kg, twice-daily for 14 days) starting on day 21 from the first STZ injection. Cytokine protein content, evaluated by ELISA, was reported as pg/mg of protein. Cytokine mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in CTR animals. Data represent mean \pm SEM of 4-6 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$ vs CTR; ^o $p < 0.05$, ^{oo} $p < 0.01$ vs STZ/saline.

5.2.4 Effect of PKR blocking on peripheral inflammatory status and immune dysfunction associated to the development of diabetes

Considering the precocious involvement of the PKs system in the onset of the diabetic neuropathy it was interesting to investigate whether a preventive blocking of PKRs with the antagonist PC1 positively influenced also the course of the diabetic pathology itself, modulating the hyperglycaemic state of animals or reducing the peripheral inflammatory component which is known to be associated to diabetes status (Agrawal and Kant, 2014). Hence, we monitored over time blood glucose values in mice injected with MLD-STZ and, at the end of the preventive treatment with PC1, we measured by ELISA the plasmatic levels of insulin. Moreover, since it is known that in diabetes model that we used, the destruction of β -pancreatic cells induces an altered immune-inflammatory response characterized by a dysregulation of cytokine expression pattern in pancreas (Cnop et., 2005; Amirshahrokhi and Ghazi-Khansari, 2012), we also compared the levels of IL-1 β and IL-10 protein content in MLD-STZ mice treated with either PC1 or saline with control animals.

As reported in figure 17, after MLD-STZ administration, diabetic mice exhibited significantly high blood glucose levels compared to control mice, which gradually increased reaching the maximal pick at the end of the period of observation, i.e. 14 days after diabetes induction (panel A). Parallel, at this time point, the levels of

plasmatic insulin were drastically reduced in MLD-STZ mice respect to the basal levels of controls (panel C). Preventive PC1 administrations from day 0 to 13 after diabetes induction was not effective either in reducing high glucose levels in mice injected with MLD-STZ or in re-establishing the plasmatic insulin levels to physiological control values.

As reported in panels B and D (fig.17), 14 days after diabetes induction, a marked dysregulation of the cytokine IL-1 β and IL-10 levels was present in the pancreas of MLD-STZ mice; both cytokines appeared significantly diminished compared to the levels of controls.

Early PC1 administrations were effective in preventing the alteration of IL-1 β and IL-10 levels which occurred in the pancreas of diabetic mice, contrasting the decrease of IL-1 β (panel B) levels and significantly avoiding the reduction of IL-10 protein content (panel D).

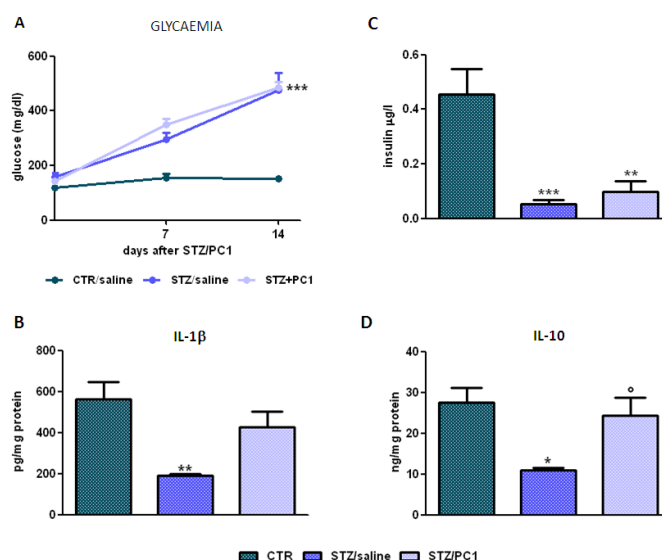


Figure 17. Time course of glycaemic values of mice injected with MLD-STZ (80 mg/kg for 3 consecutive days), s.c. administered with PC1 (150 μ g/kg, twice-daily for 14 days) or saline (preventive treatment), starting from day 0 to 13 after diabetes induction (A). Data represent mean \pm SEM of 5 mice per group. Two way ANOVA was used for statistical evaluation, followed by Bonferroni's test. *** p <0.001 vs CTR. Effect of preventive PC1 administrations on plasma levels of insulin (C) and IL-1 β and IL-10 protein content in pancreatic tissue (B, D) 14 days after diabetes induction, at the end of PC1 treatment. Plasma insulin levels and cytokine protein content, evaluated by ELISA, was reported as protein concentrations in plasma and and pg/mg of protein, respectively. Data represent mean \pm SEM of 4-6 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * p <0.05, ** p <0.01, *** p <0.001 vs CTR; $^{\circ}$ p <0.05 vs STZ/saline.

Since inflammation and immune dysregulation are important mechanisms underlying type 1 diabetes pathogenesis we finally performed ex-vivo studies evaluating the functioning of peripheral immunity at the end of preventive PC1 treatment.

In order to evaluate the effects induced by preventive PC1 administration on innate immunity, we examined the production of the cytokines IL-1 β and IL-10 by peritoneal macrophages, stimulated *in vitro* with 1 μ g/ml LPS. Figure 18 reports the concentration measured in culture supernatant of the pro-inflammatory cytokine IL-1 β and the anti-inflammatory cytokine IL-10.

The effects of the antagonist on acquired immunity were evaluated measuring Th1 and Th2 cytokine production by splenocytes, stimulated *in vitro* with 10 μ g/ml ConA. The concentration of Th cytokines IFN- γ , IL-2, IL-4 and IL-10 measured in culture supernatant is reported in figure 19.

Fourteen days after MLD-STZ injections, a significant alteration of both innate and acquired immunity was observed in diabetic mice, characterized by elevated levels of IL-1 β produced by macrophages and a Th1 pro-inflammatory/pro-cytotoxic profile.

Indeed at this time point, a significant increase of IL-1 β production by macrophages obtained from MLD-STZ mice was present, while IL-10 levels were markedly decreased, as depicted in figure 18, panels A and B, respectively. Early PC1 administrations were effective in preventing this altered balance between pro- and anti-inflammatory cytokines in MLD-STZ macrophages, maintaining the levels of IL-1 β at physiological values of controls and significantly contrasting the reduction of IL-10 (fig.18).

The Th1/Th2 cytokine balance is reported in figure 19. Fourteen days after diabetes induction, the production of Th1 cytokines increased in diabetic animals. In particular we observed a marked augmentation of IFN- γ levels (panel A) and, even if not significant, higher concentration of the cytokine IL-2 compared to controls (panel B). A protective effect induced by precocious PC1 treatment was strongly evident on IFN- γ production. As shown in panels A and B, repeated administrations of the antagonist from day 0 to 13 significantly prevented the increase of Th1 cytokines in diabetic mice, maintaining cytokine levels to basal values of control animals.

The production of IL-4 and IL-10 Th2 cytokines was not significantly altered after either MLD-STZ administrations or chronic PC1 treatment, as illustrated in panels C and D (fig.19).

IFN- γ /IL-4 ratio is frequently used as index for determining spleen cells profile. As shown in panel E (fig.19), PC1 administrations completely prevented the alteration of Th1/Th2 balance in diabetic mice, which was significantly shifted toward a Th1 phenotype 14 days after MLD-STZ injections.

Spontaneous production of cytokines by macrophages and spleen cells was always very low, at the limit of detection, and no effect of PC1 treatment was ever observed (data not shown).

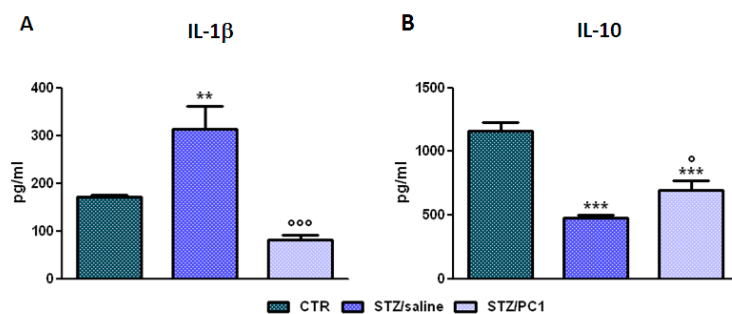


Figure 18. Effect of preventive PC1 administrations (s.c. 150 μ g/kg, twice-daily for 14 days) on IL-1 β (A) and IL-10 (B) production by peritoneal macrophages, obtained from mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) 14 days after diabetes induction, at the end of PC1 treatment. Cytokine content, evaluated by ELISA, was reported as protein concentrations in culture media. Peritoneal macrophages obtained at the end of treatment were stimulate in vitro with 1 μ g/ml LPS. Culture media were collected after 24 h. Data represent mean \pm SEM of 6-8 replicates per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. ** p <0.01, *** p <0.001 vs CTR; ° p <0.05, °°° p <0.001 vs STZ/saline.

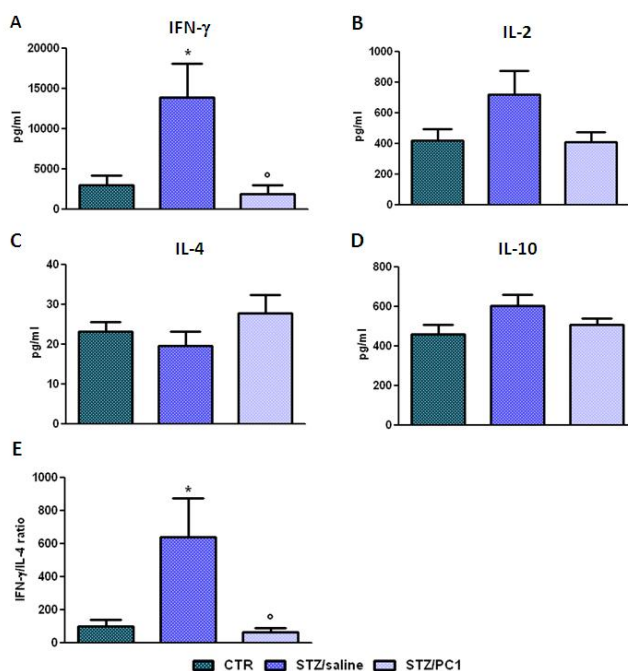


Figure 19. Effect of preventive PC1 administrations (s.c. 150 μ g/kg, twice-daily for 14 days) on IFN- γ (A), IL-2 (B), IL-4 (C) and IL-10 (D) production by splenocytes, obtained from mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) 14 days after diabetes induction, at the end of PC1 treatment. (E) Change of Th1/Th2 balance in MLD-STZ mice at the end of preventive treatment with PC1. Cytokine content, evaluated by ELISA, was reported as protein concentrations in culture media. Splenocytes obtained at the end of PC1 treatment were stimulated in vitro with 10 μ g/ml ConA. Culture media were collected after 24 h for Th1 (IL-2 and IFN- γ) and 48 h for Th2 (IL-4 and IL-10) cytokine evaluation. Splenocytes profile was expressed as IFN- γ /IL-4 ratio and calculated by taking IFN- γ /IL-4 ratio of the control as 100%. Data represent mean \pm SEM of 4-6 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * p <0.05 vs CTR; ° p <0.05 vs STZ/saline.

The functioning of peripheral immunity was ultimately investigated in MLD-STZ mice also at the end of therapeutic treatment with PC1, 35 days after diabetes induction.

As parameters of innate and adaptive immunity, we always evaluated IL-1 β and IL-10 cytokine production by peritoneal macrophages stimulated *in vitro* with 1 μ g/ml LPS, and Th1 and Th2 cytokine release by splenocytes stimulated *in vitro* with 10 μ g/ml ConA, respectively.

As depicted on figures 20 and 21, 35 days after MLD-STZ injections, both innate and acquired immunity were still altered in diabetic mice.

Therapeutic PC1 administrations from day 21 to 34 after diabetes induction efficaciously contrasted the high levels of IL-1 β produced by macrophages of diabetic mice, re-establishing cytokine production to physiological values (fig.20, panel A). IL-10 production was not significantly altered after either MLD-STZ or PC1 administration compared to controls (fig.20, panel B). We did not find any statistically significant differences for Th1/Th2 cytokine in any of the groups studied (fig.21, panels A-D). However, when we calculated the IFN- γ /IL-4 ratio as indicator of Th1/Th2 balance a significant shift towards Th1 was evident (panel E). Once again chronic treatment with the antagonist was effective against the diabetes-induced peripheral inflammatory status.

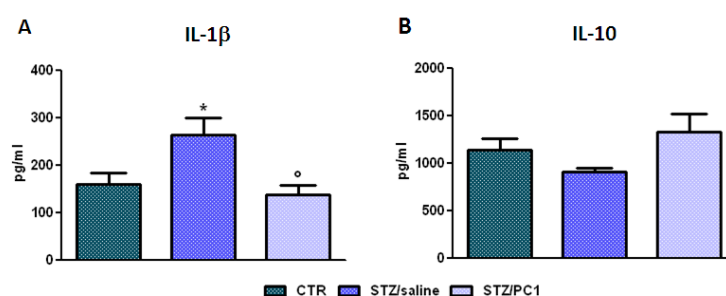


Figure 20. Effect of therapeutic PC1 administrations (s.c. 150 μ g/kg, twice-daily for 14 days) on IL-1 β (A) and IL-10 (B) production by peritoneal macrophages, obtained from mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) 35 days after diabetes induction, at the end of PC1 treatment. Cytokine content, evaluated by ELISA, was reported as protein concentrations in culture media. Peritoneal macrophages obtained at the end of treatment were stimulate *in vitro* with 1 μ g/ml LPS. Culture media were collected after 24 h. Data represent mean \pm SEM of 6-8 replicates per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * p <0.05 vs CTR; ° p <0.05 vs STZ/saline.

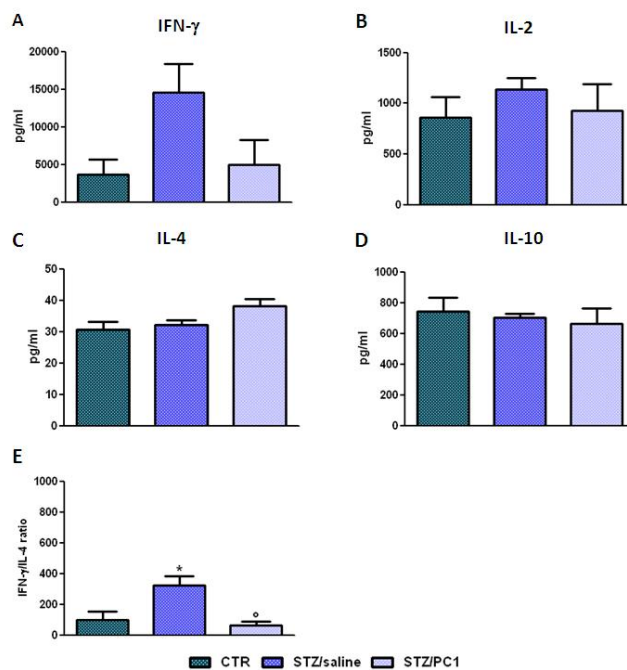


Figure 21. Effect of therapeutic PC1 administrations (s.c. 150 $\mu\text{g}/\text{kg}$, twice-daily for 14 days) on IFN- γ (A), IL-2 (B), IL-4 (C) and IL-10 (D) production by splenocytes, obtained from mice injected with MLD-STZ (80 mg/kg for 3 consecutive days) 35 days after diabetes induction, at the end of PC1 treatment. (E) Change of Th1/Th2 balance in MLD-STZ mice at the end of therapeutic treatment with PC1. Cytokine content, evaluated by ELISA, was reported as protein concentrations in culture media. Splenocytes obtained at the end of PC1 treatment were stimulated in vitro with 10 $\mu\text{g}/\text{ml}$ ConA. Culture media were collected after 24 h for Th1 (IL-2 and IFN- γ) and 48 h for Th2 (IL-4 and IL-10) cytokine evaluation. Splenocytes profile was expressed as IFN- γ /IL-4 ratio and calculated by taking IFN- γ /IL-4 ratio of the control as 100%. Data represent mean \pm SEM of 5 mice per group. One way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. * $p < 0.05$ vs CTR; ° $p < 0.05$ vs STZ/saline.

6. DISCUSSION and CONCLUSIONS

Neuropathic pain resulting from a damage or disease within the somatosensory system is a chronic pain largely resistant to treatment mainly because the underlying mechanisms are still poorly understood.

Much of the initial research on the neuropathic pain pathogenesis focused on the properties of neurons following a nerve injury, leading to proposal of both peripheral and central sensitization as important disease mechanisms (Sacerdote et al., 2013). However, in the last years it has emerged that the development and maintenance of neuropathic pain is not confined to the altered activity of sensory neurons and the reorganization of central nociceptive circuits, but also involves pathological interactions between neurons, glia and inflammatory immune cells, as well as a wide cascade of pro- and anti-inflammatory cytokines (Austin and Moalem Taylor, 2010; Calvo et al., 2012).

In the present work we provide evidences about the involvement of a recently discovered chemokine-like protein named prokineticin 2 (PK2) and its receptors PKR1 and PKR2, in the inflammatory events-related to neuropathic pain development, which occurs in the peripheral and central nervous system following neuronal injury induced either directly through a peripheral nerve lesion or as a consequence of a persistent hyperglycaemic status (diabetic painful neuropathy).

PERIPHERAL NERVE INJURY (CCI) MODEL

We initially investigated the involvement of the PKs system in a mouse model of neuropathic pain induced by the chronic constriction injury (CCI) of the sciatic nerve.

The implication of the PKs system in CCI-induced neuropathic pain was clearly confirmed by our behavioural results. In fact, blocking PK2/PKR signalling with PC1 was effective in controlling neuropathic painful manifestations, completely reverting thermal hyperalgesia and significantly reducing mechanical allodynia. The changes in pain behaviour well correlated with the expression and distribution of PK2 and PKRs at the peripheral and central sites of pain transmission.

In fact, we found a general up-regulation of PKRs and their ligand PK2 both in periphery and in spinal cord of CCI neuropathic mice.

Ten days after nerve ligation, we observed a strong increase of PK2 mRNA in the injured sciatic nerve. PK2 immunoreactivity was increased in neuroma and in nerve fibres proximal to the lesion and was associated to activated Schwann cells and infiltrating neutrophils and macrophages. In the same cells we detected high levels of both PKRs. In particular, immunofluorescence staining of the neuroma revealed a co-localization of PKR2 signal with activated Schwann cells, whereas PKR1 signal was associated to infiltrating immune cells.

The fact that few evidences indicate PKR2 as the only inducible receptor (Kisliouk et al., 2005), led us to speculate that the augmentation of PKR1 mRNA expression measured

in the injured nerve of CCI mice could be attributed to infiltrating inflammatory cells expressing this receptor, i.e. invading granulocytes and macrophages.

As previously reported in a model of inflammatory pain, the release of PK2 in the nerve contributes in lowering nociceptor activation thresholds and in recruiting neutrophils and macrophages (Giannini et al., 2009), which PK2 drive towards a pro-inflammatory phenotype increasing the release of IL-1 β and reducing that of IL-10 (Martucci et al., 2006).

Inactivation of PKRs, particularly PKR1, which is known to be the major receptor implicated in the immunomodulatory activity of PK2, appeared to be of therapeutic benefit in controlling inflammatory process which occurred in the nerve following lesion. Indeed, in the injured sciatic nerve of neuropathic CCI/PC1 mice the PK2 mRNA levels and immunofluorescence were significantly lower compared to CCI/saline group. In particular, a significant reduction of PK2 signal was detected in invading immune cells. Repeated PC1 administrations significantly reduced the availability of a potent pro-nociceptive/pro-inflammatory agent as PK2 in the nerve and in infiltrating cells, consequently preventing/reducing the further recruitment of inflammatory cells as well as the activation of resident cells. This peculiar effect of PC1 to counteract the overexpression of PK2 seems to be exclusively mediated by the activation of the PKR1 as it was demonstrated a specific involvement of this receptor in setting the enhanced PK2 levels during inflammation (Giannini et al., 2009; Negri and Lattanzi, 2011).

The activation of PK2 in the injured nerve of neuropathic mice also well correlated with the stimulation of the pro-inflammatory cytokine IL-1 β , an inflammatory mediator known to strongly contribute in enhanced pain transmission. Parallel with IL-1 β augmentation we found a significant reduction of IL-10 protein levels, whereas its mRNA expression strongly increased.

The mismatch between the mRNA and the protein levels of this anti-inflammatory cytokine is probably due to the activation of the synthetic machinery of IL-10 in order to supply the strong demand and consumption of IL-10 required for contrasting the pro-inflammatory cascade induced by the lesion (Sacerdote et al., 2013).

These data suggest that upon PK2 exposure stimulated- Schwann and immune cells produce large quantity of inflammatory mediators, including the PK2 its self, perpetuating a vicious positive feedback loop for increasing further production of inflammatory mediators, thus potentiating nerve damage and contributing to enhanced pain transmission.

Ten days after nerve ligation, PK2 signal also increased in the superficial and in some deeper layers of the spinal dorsal horns as well as in activated astrocytes. The particular increase of PK2 at the levels of the presynaptic terminals led us to suggest that PK2 may be transported to the central endings where it induces central sensitization through CGRP and SP synthesis and release (DeFelice et al., 2012),

activation of glutamate interneurons (Yuill et al., 2007) and reduction of GABA_A receptor function (Ren et al., 2011).

The expression of PKR1 was not altered in the spinal cord of CCI neuropathic mice and was restricted to resting and activated astrocytes; on the contrary neuronal injury induced a strong modulation of PKR2. As PK2, in fact the PKR2 signal clearly increased after nerve ligation in the activated astrocytes and in neuronal cell bodies, indicating a relevant role of PK2/PKR2 pair in spinal pain processing.

The PK2 released in spinal cord by activated astrocytes and primary sensory neurons may activate the PKR2 constitutively expressed in the spinal cord neurons and up-regulated after nerve injury. PKR2 was in fact increased in all examined tissues 10 days after nerve damage. Moreover, as PKR2 is primarily expressed in medium-large DRG neurons which also contain TRPA1 channel, (Negri and Lattanzi, 2011) considered involved in allodynia, and in spinal neurons we hypothesize that the increased expression of PKR2 together with its agonist PK2 might have a role in the induction and maintenance of this painful symptom.

PKR2 activation induced by PK2 may also contribute to astrogliosis and production of pro-inflammatory cytokines such as IL-1 β , which in turn stimulate astrocytes to induce further PK2 production. Previous in vitro studies support the role of PK2 to promote astrocytes activation. The release of PK2 was in fact found to induce proliferation of astrocytes expressing both PKR1 and PKR2 so functioning as an astrocyte autocrine growth factor (Koyama et al., 2006).

Repeated PC1 administration was effective in reducing the neuropathy-induced overexpression of the PK2 itself also in spinal cord. At this level, astrocytes appeared a preferential target for PC1. Indeed, in CCI/PC1 mice, astrocyte PK2 immunoreactivity was evidently reduced and the increase of IL-1 β was reverted, indicating that chronic treatment with the antagonist PC1 directly controls astrogliosis.

DIABETES-INDUCED NEUROPATHIC PAIN MODEL

Considering that in patients one of the most common causes of neuropathic pain is the presence of diabetes we have considered of great interest to investigate the role of the PKs system also in neuropathic pain induced by diabetes, using the diabetic painful neuropathy model induced by chemical pancreatectomy (STZ).

Allodynia is known to be a cardinal symptom of the diabetic painful neuropathy. In this model it appeared within few days after diabetes induction, reached full development 14 days later and persisted until 56 days from the STZ administrations.

Our data demonstrate that in diabetic mice spinal cord PK2 is implicated both in the early stage of neuropathic pain development as well as in its maintenance. An over expression of PK2 in spinal cord was in fact present since the appearance of painful symptom and for all the persistence of allodynia.

To deepen the knowledge about the timing of activation and the specific role of the PKs system in diabetic neuropathic pain evolution we performed two different pharmacological approaches using the PKR antagonist PC1. Mice were chronically treated with PC1 at different time points from diabetes induction, i.e. either starting on day 21 after diabetes induction, when animal exhibited an overt neuropathic pain (therapeutic treatment) or at the same time of STZ administrations (day 0), i.e. when hyperglycaemia and mechanical allodynia were not yet developed (preventive protocol).

The antagonist PC1 was highly effective in alleviating diabetes-induced neuropathic pain. In fact repeated administrations of PC1 significantly reduced mechanical allodynia in STZ mice treated in presence of fully developed neuropathic pain, while completely prevented the development of painful symptoms, when it was given to animal not already neuropathic. Interestingly, therapeutic treatment schedule delayed painful symptom reappearance after PC1 suspension, leading us to suppose that blocking PK2 signalling could induce permanent changes in the neuronal circuits or in the neuroinflammatory phenomena involved in the maintenance of neuropathic pain. Moreover, a single bolus systemic injection of PC1 rapidly reduced the established STZ-induced allodynia in mice with fully developed neuropathic pain, suggesting a direct action on nociceptor PKRs, whose blockade hinders the transmission of painful stimuli. The clear anti-allodynic effect observed after chronic PC1 treatment could be also ascribed to its ability to prevent or reduce the PK2 synthesis activation. Consistently, 35 days after diabetes induction PK2 and PKR2 were significantly increased in spinal cord of STZ mice and therapeutic PC1 administrations reduced these augmentations; the counteraction of the PK2 overexpression was even more evident after the precocious treatment. In addition, the prevention of spinal cord PK2 up-regulation was long lasting and persisted after suspension of PC1 administrations.

The observation that in mice precociously treated with PC1 administrations allodynia did not develop led us to hypothesize that when PC1 treatment started on day 0, i.e. when the unique plasticity of the central nervous system that underlies allodynia was not yet developed, the blocking of neuronal PKR2 and PK2 synthesis in spinal cord could prevent or slow this neuronal plasticity.

In order to further support the anti-allodynic effect of PC1, we also analysed the expression of glutamate NMDA receptor subunits N1, N2A and N2B and the glutamate AMPA receptor subunits A1 and A2/3 in spinal cord of STZ mice treated with preventive PC1 administrations.

As reported in literature, the development of abnormal pain perception in diabetic animals is associated with abnormal hyperactivity of glutamate receptors in spinal cord. In fact, it was found that hyperalgesia and allodynia exhibited by diabetic mice can be prevented by co-administrations of AMPA and NMDA receptors antagonists (Calcutt and Chaplan, 1997; Gupta et al., 2003; Malcangio and Tomlinson, 1998).

Further evidences indicate that the alterations in synaptic transmission associated to neuropathic pain development include phosphorylation of NMDA receptors, altered NMDA receptor subunit expression pattern and an increase in NMDA-mediated current (Guo et al., 2002; Zou et al., 2002; Gaunitz et al., 2002; Karlsson et al., 2002; Isaev et al., 2000). In particular pain hypersensitivity induced by peripheral injury or tissue inflammation is known to be mediated by the activation of N2B subunits distributed in spinal cord dorsal horns (Boyce et al., 1999; Sakurada et al., 1998; Tan et al., 2005; Taniguchi et al., 1997) Moreover, Iwata et al. (Iwata et al., 2007) demonstrated that on spinal neurons the incidence of N2A decreased while that of N2B increased after peripheral nerve injury.

In agreement with this literature, in presence of fully developed mechanical allodynia a decrease of the spinal NMDA receptor subunit N2A was present, while the expression of the N2B subunit significantly increased. Early PC1 administrations were effective in preventing N2B up-regulation in spinal cord of diabetic mice, without affecting the levels of the subunit N2A.

These data confirm that PC1 administration has a clear impact on neuronal processes that participate in the establishment of allodynia.

As already discussed, the PKs system plays a pivotal role in modulating peripheral immune/inflammatory reactions as well as neuroinflammation. As further confirm, we demonstrated that PC1 treatment was able to significantly reduce IL- β overexpression in the spinal cord in STZ mice. As for the CCI results, it can be suggested that by reducing PK2 and PKR2 levels in spinal cord, the signalling leading to IL-1 β production by astrocytes and microglia is significantly blunted.

From our data, it also emerges an important control of PK2 on the balance of pro- and anti-inflammatory cytokines in the sciatic nerve. In the diabetic nerve, Schwann cells, resident and macrophages recruited from bloodstream present a clear pro-inflammatory phenotype, characterized by high IL-1 β and low IL-10 levels. The antagonism with PC1 was efficacious in reverting this pro-inflammatory phenotype; indeed after treatment we measured extremely high levels of sciatic nerve IL-10, suggesting that the blocking of PKRs drives nerve macrophages towards an anti-inflammatory direction. As previously addressed for CCI, the augmentation of PKR1 mRNA expression measured in the diabetic nerve could be attributed to infiltrating inflammatory cells expressing this receptor. Since PKR1 is the most implicated in the immune response and it was previously demonstrated to mediate macrophage chemotaxis (Martucci et al., 2006) it can be assumed that blocking it with PC1 could affect macrophage migration reducing or preventing the recruitment of further inflammatory cells expressing PKR1 in the nerve.

Inflammation and immune activation have been recognized as fundamental mechanisms in the pathophysiology of diabetes as well as of its complications (Agrawal and Kant, 2014). An autoimmune reactivity characterized by a T helper 1 profile is

consistently present in diabetes, and elevated pro-inflammatory cytokines, such as IFN- γ , TNF- α , and IL-1 β (Padgett et al., 2013) also participate in pancreatic β -cells destruction. In agreement with our and other groups' studies about the potent immunomodulatory activity of PK2 (Martucci et al., 2006; Franchi et al., 2008, LeCouter et al., 2004), we now demonstrate that PK2 and its receptors appear to be involved also in modulating the altered peripheral immune response in the STZ model. In fact, in diabetic mice we observed a significant alteration of both innate and acquired immunity, characterized by elevated levels of IL-1 β produced by circulating macrophages, and a Th1 pro-inflammatory/pro-cytotoxic profile. PC1 treatment reduced the peripheral inflammatory status, decreasing macrophagic IL-1 β and switching Th1/Th2 balance towards Th2. Interestingly, blocking PKRs was also able to prevent cytokine alteration that we observed in pancreas. At the moment we do not know whether this effect is mediated by PKRs that have been shown to be present on pancreatic tissues (Jiang et al. 2009; Cline et al., 2011; Dormishian et al., 2013), or it depends on the general peripheral immunomodulation achieved with PC1.

We can hypothesize that PK2 is involved in all the inflammatory processes that take place in diabetes, and the inactivation of its signalling might ameliorate the general well-being in the animals, as demonstrated by the reduction of painful symptoms. If this hypothesis is true, the blocking of PKRs could have beneficial effects also on other diabetic complications, such as retinopathy and nephropathy. Further experiments are needed in this direction.

Although PC1 blocked the STZ-induced neuropathic pain, neuroinflammation and peripheral immune activation, it did not affect hyperglycaemia nor the body weight loss present in diabetic mice. However this is not uncommon, since other compounds, such cannabis extracts, buprenorphine and ghrelin, have beneficial action on diabetic alterations and tissue damage without affecting STZ-induced hyperglycaemia and body weight loss (Saini et al., 2007; Comelli et al., 2009; Canta et al., 2009; Kyoraku et al., 2009). Interestingly, also the STZ diabetic rats treatment with low dose insulin normalizes tactile allodynia, without affecting hyperglycaemia (Hoybergs and Meert, 2007). We believe that in the whole, PK2 has a role in the several inflammatory events that take place downstream of hyperglycaemia.

CONCLUSIONS

Blocking PK2 signalling with PC1 appears a winning strategy for controlling neuropathic pain as this molecule not only significantly reduces neuropathy-induced pain hypersensitivity directly targeting the receptors, but also suppresses the causes underlying disease progression as neuroinflammation, by controlling the synthesis and release of the pro-inflammatory/pro-nociceptive endogenous PK2.

In conclusion, we suggest the PKs system as a promising target for the development of novel pharmacological approaches to treat neuropathic pain as well as other pathological conditions characterized by a sustained inflammatory component.

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8. LIST OF PUBLICATIONS

LIST OF PUBLICATIONS:

Castelli M, Panerai A, Sacerdote P, Franchi S. (2015) *Measurement of macrophage toll-like receptor 4 expression after morphine treatment*. *Methods Mol Biol.* 1230:263-71.

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- PAPER IN PREPARATION

Castelli M, Amodeo G, Negri L, Lattanzi R, Gotti C, Panerai AE, Sacerdote P, Franchi S. *The antagonism of the prokineticin system prevents and reverses the inflammatory status and neuropathic pain in a mouse model of diabetes*. *Diabetologia*.

ABSTRACTS:

Castelli M, Franchi S, Amodeo G, Lattanzi R, Negri L P. Sacerdote, Panerai AE. *The antagonism of PKs system contrasts neuropathic pain and the inflammatory status in a mouse model of diabetes.*

17th SIF meeting, Rimini September 16-18 2014

Castelli M, Moretti S, Franchi S, Balboni G, Lattanzi R, Negri L, Panerai AE, Sacerdote P. *Role of a new family of chemokines, the prokineticins, in experimental neuropathic pain.*

6th SIMPAR meeting, Roma March 28-29 2014

Castelli M, Moretti S, Franchi S, Balboni G, Lattanzi R, Negri L, Panerai AE, Sacerdote P. *Precocious involvement of prokineticin system in diabetic neuropathic pain in mice.*

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Castelli M, Moretti S, Franchi S, Lattanzi R, Negri L, Panerai AE, Sacerdote P. *Role of the prokineticin system in different models of neuropathic pain.*

Next Step 4:la giovane ricerca avanzata, Milan July 17 2013

Castelli M, Moretti S, Franchi S, Balboni G, Lattanzi R, Negri L, Panerai AE, Sacerdote P. *The antagonism of the prokineticin system reverses diabetic neuropathic pain and restores cytokine balance in mice.*

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Franchi S, **Castelli M**, Moretti S, Panerai AE, Sacerdote P. *Stem cells as advanced therapy of neuropathic pain.*

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