Department of Physiology and Pharmacology “V Erspamer”

DOCTORAL RESEARCH IN
PHARMACOLOGY AND TOXICOLOGY
XXXI CYCLE

Novel promising pharmacological targets and drugs for the treatment of neuropathic pain: focusing on prokineticin system and selective A3 adenosine receptor (A3AR) agonists in an animal model

Supervisor:
Prof. ROBERTA LATTANZI

PhD Candidate:
SILVIA SQUILLACE

Referees:
Prof. DANIELA SALVEMINI
Prof. ROSSELLA MIELE

A/A 2017-2018

SUMMARY

When things go wrong, as they sometimes will,
When the road you're trudging seems all uphill,
When the funds are low and the debts are high,
And you want to smile, but you have to sigh,
When all is pressing you down a bit,
Rest if you must, but don't you quit.

Success is failure turned inside out,
The silver tint on the clouds of doubt,
And you can never tell how close you are,
It may be near when it seems far.

So stick to the fight when you're hardest hit,
It's when things go wrong that you must not quit.
- *J. G. Whittier*

"All the pieces will fit together"
- *Deno Fabbre*

1. INTRODUCTION

Pain is described as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. This definition was promulgated by the International Association for the Study of Pain (IASP) and it is in constant updating since its first publication (Merskey, 1979). Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life. It is unquestionably a sensation in a part or more parts of the body and it is always unpleasant and, therefore, it is also an emotional experience. Pain motivates the individual to withdraw from damaging situations, to protect a damaged body part while it heals, and to avoid similar experiences in the future. The inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment.

Pain should not be confused with nociception (Loeser and Treede, 2008). Nociception refers to the peripheral and central nervous system (CNS) processing of information about the internal or external environment, as generated by the activation of nociceptors. Typically, noxious stimuli, including tissue injury, activate nociceptors that are present in peripheral structures and that transmit information to the spinal cord dorsal horn or its trigeminal homologue, the nucleus caudalis. From there, the information continues to the brainstem and ultimately the cerebral cortex, where the perception of pain is generated (Fig 1). Hence, pain is a product of higher brain center processing and it is defined as a subjective experience that arguably exists only in the person that feels it (first-person perspective). Nociception is defined as observable activity in the nervous system in response to an adequate stimulus (third-person perspective) (Davis et al., 2017). The distinction between nociception and pain is also important for behavioural studies in which an understanding of pain mechanisms is the ultimate goal. Many behavioural tests involve assessment of reflex responses to noxious stimuli, typically applied at threshold or just suprathreshold intensities to incite a brief withdrawal of the tail or paw. These are principally tests of nociceptive processing because stimulus duration is limited by the animal’s response. On the other hand, the endpoints of more complex behaviours are presumed to involve supraspinal areas of the brain and as such are tests of both nociception and pain.

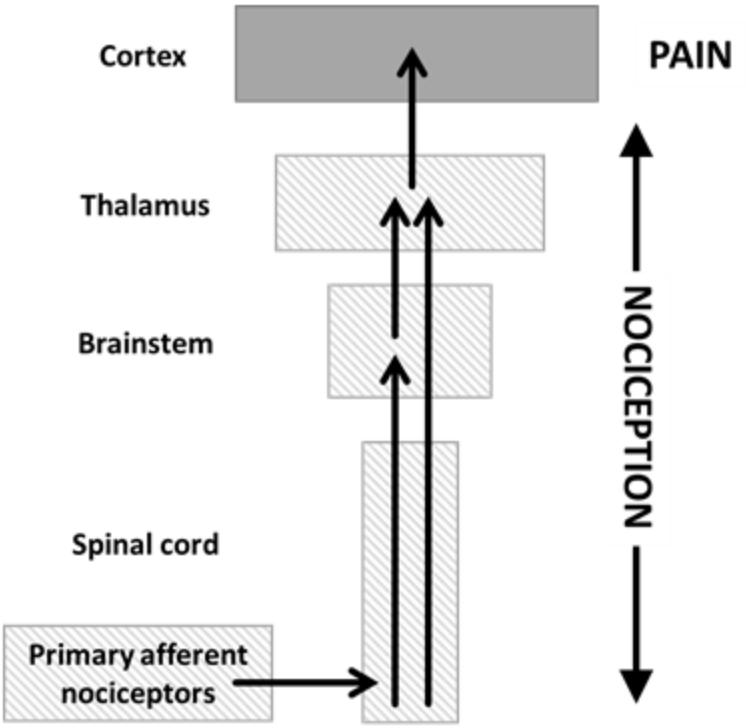

Fig 1. Anatomical distribution of nociception and pain. This figure schematizes the major neuroanatomical structures that differentiate nociception and pain, an understanding of which is essential for studies in which the animals may experience pain. Nociception refers to the process through which information about peripheral stimuli is transmitted by primary afferent nociceptors to the spinal cord, brainstem, thalamus, and subcortical structures. In contrast, the experience of pain can result only when there is activity of thalamocortical networks (represented in the dark shaded box at the top) that process the information conveyed by pathways of nociception. The magnitude of pain is determined to a great extent by the strength of descending inhibitory and facilitatory controls (in the lighter shaded boxes) that originate throughout the neuraxis and regulate the processing of ascending nociceptive messages [Figure adapted from (National Research Council Committee, 2009)].

Pain can be classified according to its location (e.g. visceral, joint or back pain...), to the clinical context (e.g. postsurgical, malignancy related, psychogenic...), to its intensity (mild, moderate, severe), and all of these categories sometimes overlap. However, the first, and probably the most intuitive, classification of pain was based on its duration: a pain sensation that lasts for less than 30 days is defined as acute pain. It has a protective role, acts as an alarm for the organism and it is essential for survival. Chronic pain, instead, has been recognized as pain that persists past normal healing time (Bonica, 1953) and hence lacks the acute warning function of physiological nociception (Treede, 2011). Usually pain is regarded as chronic when it lasts or recurs for more than 3 to 6 months (Merskey, 1994). Chronic pain is a frequent condition, affecting an estimated 20% of people worldwide (Breivik et al., 2006; Goldberg and McGee, 2011; Treede et al., 2015) and accounting for 15% to 20% of physician visits (Koleva et al., 2005). Chronic pain should receive greater attention as a global health priority because adequate pain treatment is a human right, and it is the duty of any health care system to provide it (Goldberg and McGee, 2011).

1.1 Neuropathic pain

Neuropathic pain elicited by damage to or dysfunction of the sensory nervous system severely affects quality of life and is associated with a high economic cost for both the individual and society (Attal et al., 2011; Finnerup et al., 2015). The symptoms of neuropathic pain are sensory hypersensitivity defined by spontaneous pain, hyperalgesia, and allodynia (Jensen et al., 2011; Jensen and Finnerup, 2014) resistant to standard analgesics (Finnerup et al., 2010). Hyperalgesia is defined as an abnormally increased sensitivity to pain, whereas allodynia is a painful sensation caused by innocuous stimuli like a light touch.

Neuropathic pain can be caused by not only physical lesions (e.g., traumatic nerve injury and spinal cord injury) but also other reasons, such as diabetes, chemotherapy, and viral infection (Baron et al., 2010). It is difficult to treat all types of hypersensitivity with currently available medications, and many patients with neuropathic pain do not receive appropriate treatment (Attal and Bouhassira, 2015; Torrance et al., 2013). Thus, there is an urgent need for evidence-based development of novel pharmacotherapies for neuropathic pain.

Growing evidence suggests that neuropathic pain induced by nerve damage is caused by a process of chronic inflammation. Upon nerve injury, damaged cells secrete pro-inflammatory molecules that activate cells in the surrounding tissue and recruit circulating leukocytes to the site of injury. Among these, the most abundant cell type is macrophages, which produce several key molecules involved in pain enhancement, including cytokines and chemokines. Given their central role in the regulation of peripheral sensitization, macrophage-derived cytokines and chemokines could be useful targets for the development of novel therapeutics. Inhibition of key pro-inflammatory cytokines and chemokines prevents neuroinflammation and neuropathic pain. In the following sections, the contribution of immune system in the onset and maintenance of neuropathic pain will be discussed more in detail.

1.1.1 Pathophysiology of neuropathic pain: the role of immune system

Recent studies have suggested that interactions between the nervous and immune systems trigger chronic neuroinflammation resulting in aberrant sensory processing and neuropathic pain (Calvo et al., 2012; Ji et al., 2016). Upon nerve injury, several cell types, including damaged neurons, demyelinated Schwann cells, and tissue-resident macrophages, produce soluble inflammatory cytokines, chemokines, and damage-associated molecular patterns (DAMPs) that activate surrounding cells (Thacker et al., 2007; Zhang et al., 2016) and recruit circulating leukocytes, such as monocytes/macrophages, neutrophils, and lymphocytes, into the site of injury (Kiguchi et al., 2012;

Ren and Dubner, 2010). Numerous pro-inflammatory cytokines (e.g., IL-1β and TNFα) and chemokines (e.g., CCL2, CCL3 and CCL4) are released by the infiltrating leukocytes, directly sensitize nociceptors, and alter the processing of nociceptive information by sensory neurons (Nicol et al., 1997; Oh et al., 2001).

Peripheral and Central sensitization

It is well known that pain sensation is processed by a discriminative set of primary afferent neurons (Basbaum et al., 2009; Todd, 2010). Unmyelinated C-fibers and thinly myelinated Aδ fibers act as nociceptors, while myelinated Aβ fibers are tactile sensors (Dubin and Patapoutian, 2010). Noxious stimuli such as heat, cold, pressure, and chemicals are converted to electrical activity by distinct cation channels (e.g., transient receptor potential (TRP) channels and sodium channels) that elicit action potentials (Moran et al., 2011; Waxman and Zamponi, 2014). Primary afferent neurons producing glutamate or neuropeptides transmit peripheral information to secondary neurons in the spinal dorsal horn (Basbaum et al., 2009; Todd, 2010). During neuropathic pain, the expression and sensitivity of these channels become dysregulated and elicit ectopic activity of nociceptive DRG neurons (Dib-Hajj et al., 2009; Liu and Wood, 2011). Despite the complexity of the underlying mechanisms, the close relationship between ectopic activity and pro-inflammatory mediators has been noted in several studies (Pinho-Ribeiro et al., 2017; Xanthos and Sandkuhler, 2014). Because many nociceptive DRG neurons express pro-inflammatory cytokine and chemokine receptors that are upregulated after nerve injury, pro-inflammatory molecules can directly sensitize nociceptors, such as TRP channels, in C-fibers leading to hypersensitivity. For example, IL-1β, TNFα, IL-6, CCL2, and CCL3 are well-known enhancers of nociceptor activity (Nicol et al., 1997; Oh et al., 2001). Thus, long-lasting neuroinflammation resulting from upregulation of inflammatory molecules by damaged tissue and infiltrating leukocytes can contribute to the ectopic discharge of sensory neurons, resulting in peripheral sensitization. Prolonged abnormal transmission of pain signalling into the spinal dorsal horn due to peripheral sensitization triggers central sensitization (Haroutounian et al., 2014; von Hehn et al., 2012), characterized by increased excitability of pain-processing neurons and activation of glial cells (microglia and astrocytes) (Grace et al., 2014; Ji et al., 2014). These glial cells have been the focus of increasing attention in the past few decades, and their critical contribution to spinal neuroinflammation underlying neuropathic pain is now well characterized (McMahon et al., 2005; Scholz and Woolf, 2007; Tsuda et al., 2005). Microglia and astrocytes are activated by several neurotransmitters derived from primary afferent neurons, such as cytokines, chemokines, and nucleotides. Activation of glial cells induces a variety of pro-inflammatory factors that directly or indirectly sensitize pain-processing neurons in the spinal dorsal horn (Calvo et al., 2012; Grace et al.,

2014; Ji et al., 2014). Similar to the peripheral response, typical inflammatory cytokines (IL-1β, TNFα, IL-6), chemokines (CCL2 and CCL3), and growth factors are upregulated in the dorsal horn after nerve injury, and inhibition of these molecules reverses neuropathic pain (Kiguchi et al., 2010; Thacker et al., 2009). These pain-facilitating molecules function to sensitize ionotropic glutamate receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptors (Basbaum et al., 2009; Grace et al., 2014). Because AMPA and NMDA receptors play central roles in pain processing in the spinal cord, modulation of their sensitivity by pro-inflammatory mediators derived from activated glial cells is also important for the pathogenesis of neuropathic pain (Fig. 2).

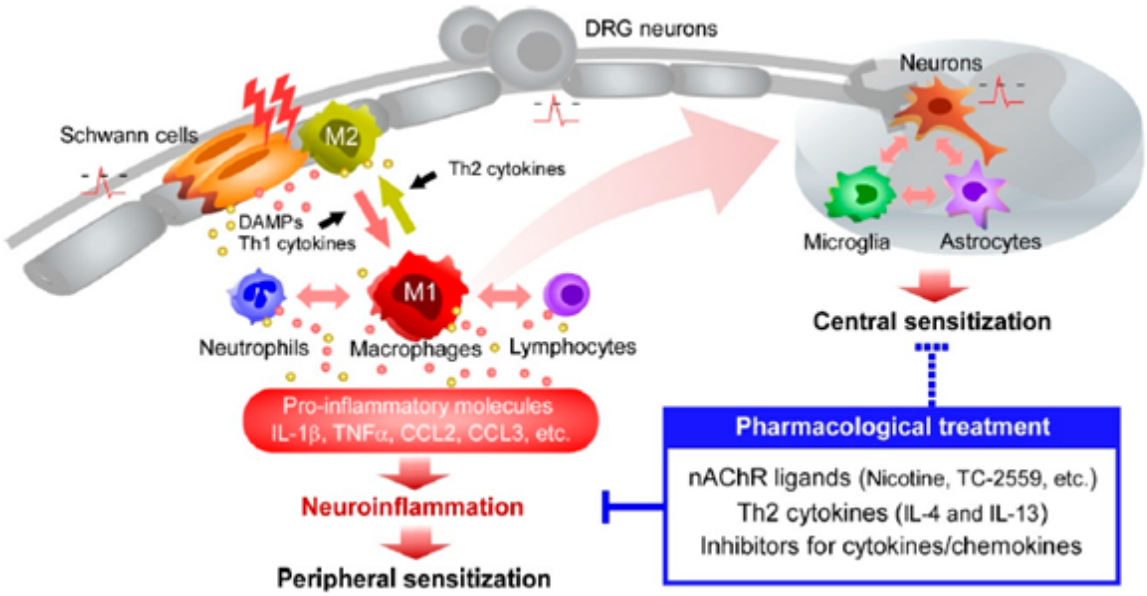

Fig 2. Generation of neuropathic pain by macrophage-driven inflammation in the peripheral nervous system. After nerve injury, activated resident cells (Schwann cells and macrophages) produce soluble factors such as damage-associated molecular patterns (DAMPs) that activate nearby cells and recruit circulating leukocytes (macrophages, neutrophils, and lymphocytes) to the site of injury. Macrophages are the most abundant infiltrating leukocyte population and are thought to play a central role in regulating peripheral neuroinflammation. Tissue-resident and infiltrating leukocytes communicate through the release of pro-inflammatory mediators such as cytokines and chemokines, which convey nociceptive information to dorsal root ganglia (DRG) neurons. Persistent ectopic activity of DRG neurons induces central sensitization characterized by the enhanced activity of pain processing neurons and the activation of microglia and astrocytes. Pharmacological targeting of macrophages or macrophage-derived pro-inflammatory molecules by nicotinic acetylcholine receptor (nAChR) ligands, Th2 cytokines, and inhibitors of cytokines and chemokines can suppress macrophage-driven neuroinflammation after nerve injury. The reduction in neuroinflammation improves both peripheral and central sensitization and alleviates intractable neuropathic pain [Source: (Kiguchi et al., 2017)].

1.1.2 Animal models of neuropathic pain

The study of neuropathic pain mechanisms is largely based on animal models (Calvo et al., 2012; Sah et al., 2003; Ueda, 2006), which date back to the late 19th century (Von Frey, 1896). Although these models have weak points, they have immensely contributed to our understanding of the key components of neuropathic pain development. Animal models of pain are designed to mimic distinct clinical diseases to better evaluate underlying mechanisms and potential treatments. Outcome measures are designed to evaluate multiple parts of the pain experience including reflexive hyperalgesia measures, sensory and affective dimensions of pain and impact of pain on function and quality of life.

Peripheral nerves have been targeted in many well characterized models of neuropathic pain. Direct nerve injury models include I) ligating or transecting the spinal nerves (spinal nerve ligation, SNL, or spinal nerve transection SNT), II) ligating or lesioning the sciatic nerve (chronic constriction injury, CCI), and III) ligating distal branches (peroneal, tibial) of the sciatic nerve (spared nerve injury, SNI) (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Kim and Chung, 1992). The principles of these methods may be applied to nerves other than the sciatic, such as orofacial nerves (Vos et al., 1994). The behavioural phenotypes are essentially indistinguishable between these different peripheral nerve models, with decreased withdrawal thresholds to mechanical and thermal stimuli and spontaneous guarding behaviour of affected limbs (Takaishi et al., 1996).

The existing models, however, are often criticized to not reflect clinical pain characteristics (Cobos et al., 2012), which are mostly of spontaneous nature. Persistent or chronic pain is experienced by day and night. It affects sociability and often the ability for voluntary behavioural tasks. Pain increases the rate, frequency, or intensity of some behaviors (eg, withdrawal responses) and suppresses other behaviors (eg, feeding). These aspects are severely under investigated in rodents and difficult to assess. While patients can describe their pain orally, most rodent studies rely on short-duration stimulus-evoked unilateral hind paw measurements. It is commonly agreed that we need to analyze new parameters that may reflect impairments in the quality of life (Barrot, 2012).

Neuropathic pain models described above induce changes also in non-reflexive (spontaneous) pain, such as vocalization (Kurejova et al., 2010), change in spontaneous motor activity (Gregoire et al., 2012), conditioned place preference (CPP), escape avoidance (PEAP) (LaGraize et al., 2004) and social behaviour such as dominance (Monassi et al., 2003). However, these tests are subject of controversy and do not work consistently across laboratories (Tappe-Theodor and Kuner, 2014). There are numerous reasons for this, including the lack of standardization.

Over the past two decades, numerous novel 'pain targets' including receptors, ion-channels and enzymes have been identified and implicated in the pathophysiology of chronic pain. However, most compounds that modulate these targets failed to show analgesic efficacy in proof-of-concept human clinical trials, despite promising preclinical data. For this reason, the research of novel drugs for the treatment of neuropathic pain have to consider the efficacy of each compound on pain-suppressed and non-evoked pain behaviours rather than only pain-elicited behaviours. This will provide means for distinguish true analgesics and will bring us closer to a better bench-to-bedside translation.

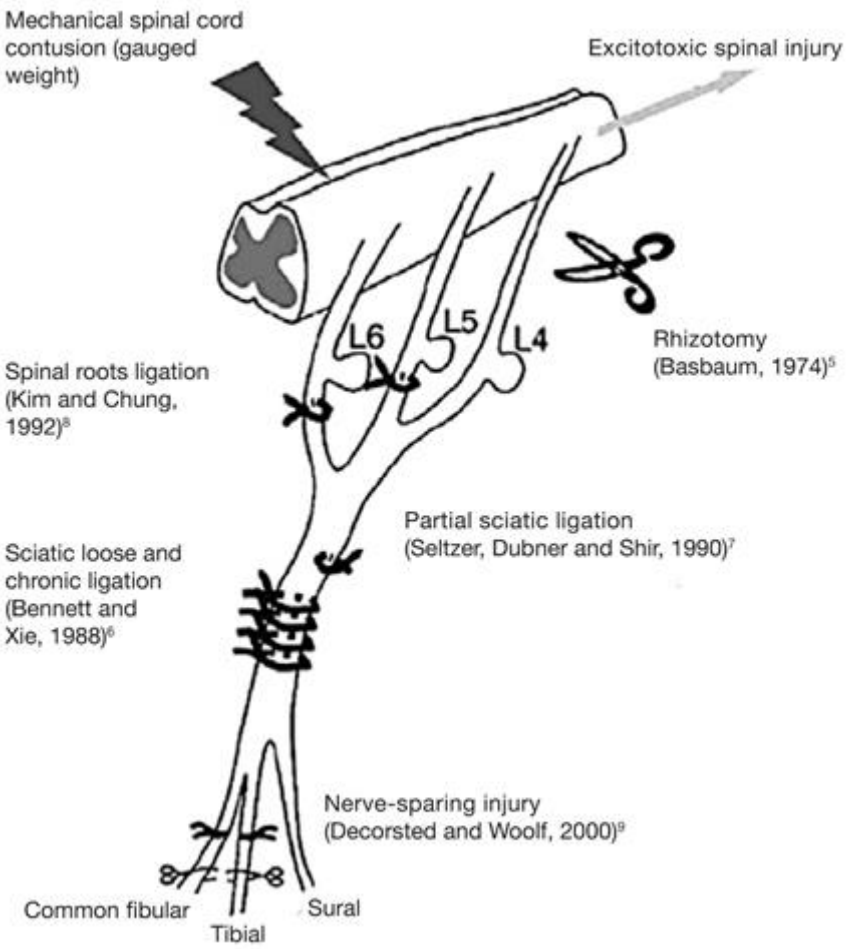

Fig 3. Experimental models of neuropathic pain. [Source (Challa, 2015)]

1.1.2.1 Chronic Constriction Injury (CCI)

Sciatic nerve CCI resembles human neuropathy resulting from trauma of peripheral nerves, with some functional preservation of the innervation (nerve entrapment or compression) (Colleoni and Sacerdote, 2010). The CCI model was first described in 1988 (Bennett and Xie, 1988) and the surgical

procedure consists of the loose ligation of the sciatic nerve at mid-thigh level with not absorbable sutures. An inflammatory reaction develops in response to the surgery and consequentially a loss of most A-fibres and some C-fibres, but few cell bodies. This is associated with spontaneous pain-related behaviour, allodynia and hyperalgesia. It has been demonstrated that anti-inflammatory treatments of CCI animals decreases the associated thermal hyperalgesia and so it is speculated that there is a significant inflammatory component in the development of the painful neuropathy (Bridges et al., 2001).

1.2 ADENOSINE PATHWAY: SAFER PAIN-RELIEVING PROSPECTS

1.2.1 Acting on adenosine signalling to modulate inflammation and pain

Adenosine, an endogenous purine nucleoside composed of adenine attached to a ribose, is constitutively present in the extracellular space (ES) and it is an important signalling molecule which regulates several biological functions. Under normal metabolic conditions, concentrations of adenosine in ES are definitively low (in the low-micromolar to high-nanomolar range) and the majority of it is taken up into cells and rapidly incorporated into ATP stores or deaminated by adenosine deaminase (Latini and Pedata, 2001). The occurrence of pathological events promotes a massive release of ATP from damaged or dying cells, and its consequent accumulation in ES. ATP released provides qualitative and quantitative information about the injury, triggering a series of proinflammatory responses including "danger" and "find me" signals for phagocytes to migrate to damaged tissue (Dosch et al., 2018; Trautmann, 2009). Extracellular ATP metabolism is mediated by a cascade of membrane-bound nucleotidases: CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1, E-NTPDase 1) converts ATP into AMP and then CD73 (ecto-5'-nucleotidase, Ecto5'NTase) dephosphorylates AMP into adenosine (Hasko et al., 2008). In this way, in response to stress or injury, the extracellular concentration of adenosine can increase up to 1000-fold (Ballarin et al., 1991) and this drives a shift from an ATP-driven proinflammatory environment to an anti-inflammatory milieu induced by adenosine (Beavis et al., 2012). Adenosine elicites responses in various cell types of the central nervous system including neurons, astrocytes, and microglia (Fredholm et al., 2011) and in general promotes, also at peripheral level, a depressive action on immune cells activity. In particular, it has been shown that adenosine enhances anti-inflammatory activity of Tregs and, at the same time, reduces the proinflammatory activity of effector T cells by inducing their differentiation in Tregs. By employing these two different mechanisms, the beneficial effects of adenosine result quickly effective and persistent (Ohta and Sitkovsky, 2014) (Fig 4).

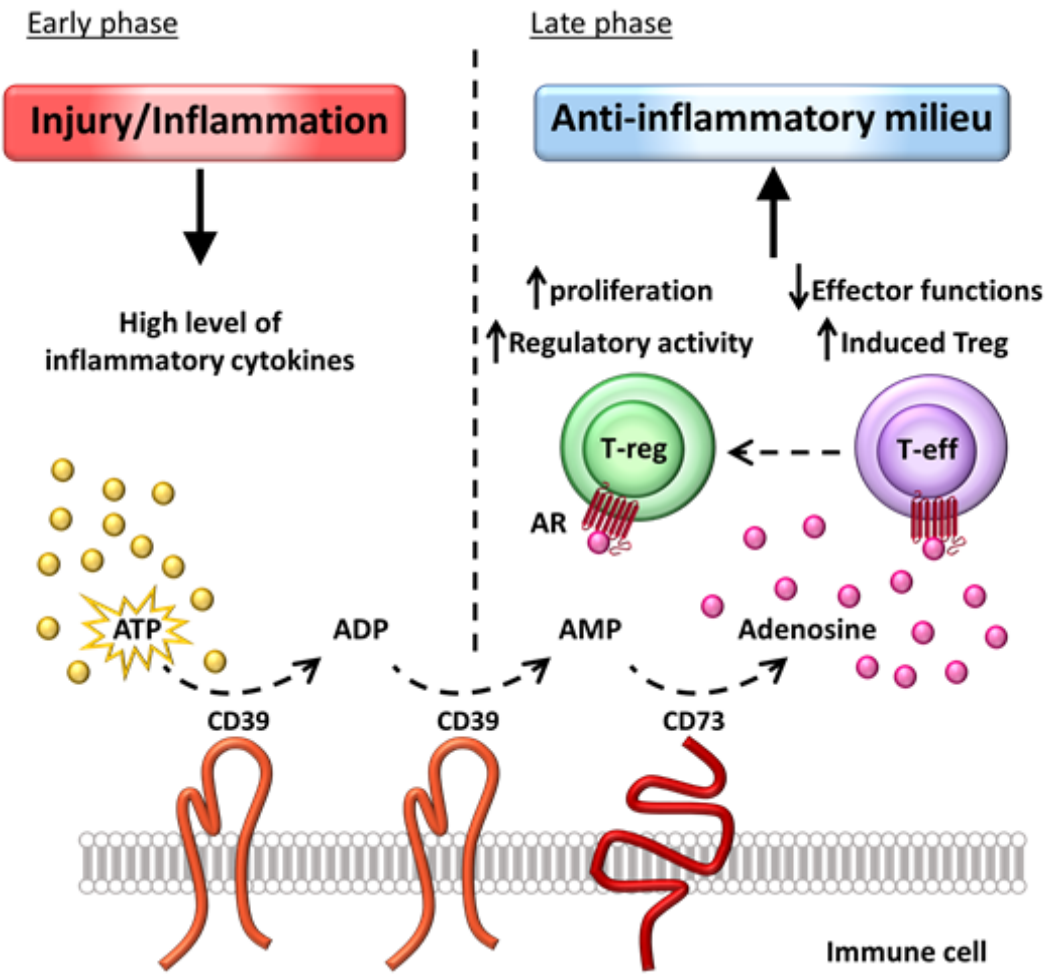

Fig 4 Adenosine promotes a depressive action on immune cells activity and exerts a potent anti-inflammatory effect. ATP released into ES from stressed or damage cells acts as an important signalling molecule. Adenosine is the final bioactive product of ATP breakdown. The increase of adenosine concentrations in ES is related to the activation of an auto regulatory loop, the function of which is to modulate immune system and inflammatory response.

Adenosine acts through a family of four G protein-coupled adenosine receptors (ARs): A_1, A_{2A}, A_{2B} and A_3 each of which has a unique pharmacological profile, tissue distribution and effector coupling. Among the human ARs, the most similar are the A_1 and A_3 ARs (49% sequence similarity) and the A_{2A} and A_{2B} ARs (59% sequence similarity). ARs have traditionally been classified based on their differential coupling to Gα subunit: A_1 and A_3 are coupled to G_i/G_q proteins while A_{2A} and A_{2B} are coupled to G_s (Fredholm et al., 2011). Therefore, activation of the A_{2A} and A_{2B} ARs subtypes increases cyclic AMP production, resulting in activation of protein kinase A (PKA) and phosphorylation of the cyclic AMP response element binding protein (CREB). In contrast, activation of the A_1 and A_3 ARs inhibits cyclic AMP production and decreases PKA activity and CREB phosphorylation (Cunha, 2001; Jacobson and Gao, 2006). In some cases, the A_1AR increases

phospholipase C (PLC) activity through a pertussis toxin-sensitive G protein. The A_1AR can also directly couple to and inhibit cardiac K^+ channels and types Q, N and P voltage sensitive Ca^{2+} channels. In turn, the A_3AR can regulate the activity of PLC via a pertussis toxin-sensitive G protein or by direct coupling to G_q protein (Jacobson and Gao, 2006; Sheth et al., 2014). All four subtypes of ARs can couple to mitogen-activated protein kinase (MAPK), giving them a role in cell growth, survival, death and differentiation (Fig 5). Phosphorylation and subsequent desensitization of ARs have been studied for all four subtypes. The rapidity of the desensitization depends on the subtype, with the A_3AR being more rapidly desensitized than the other subtypes (Olah and Stiles, 2000).

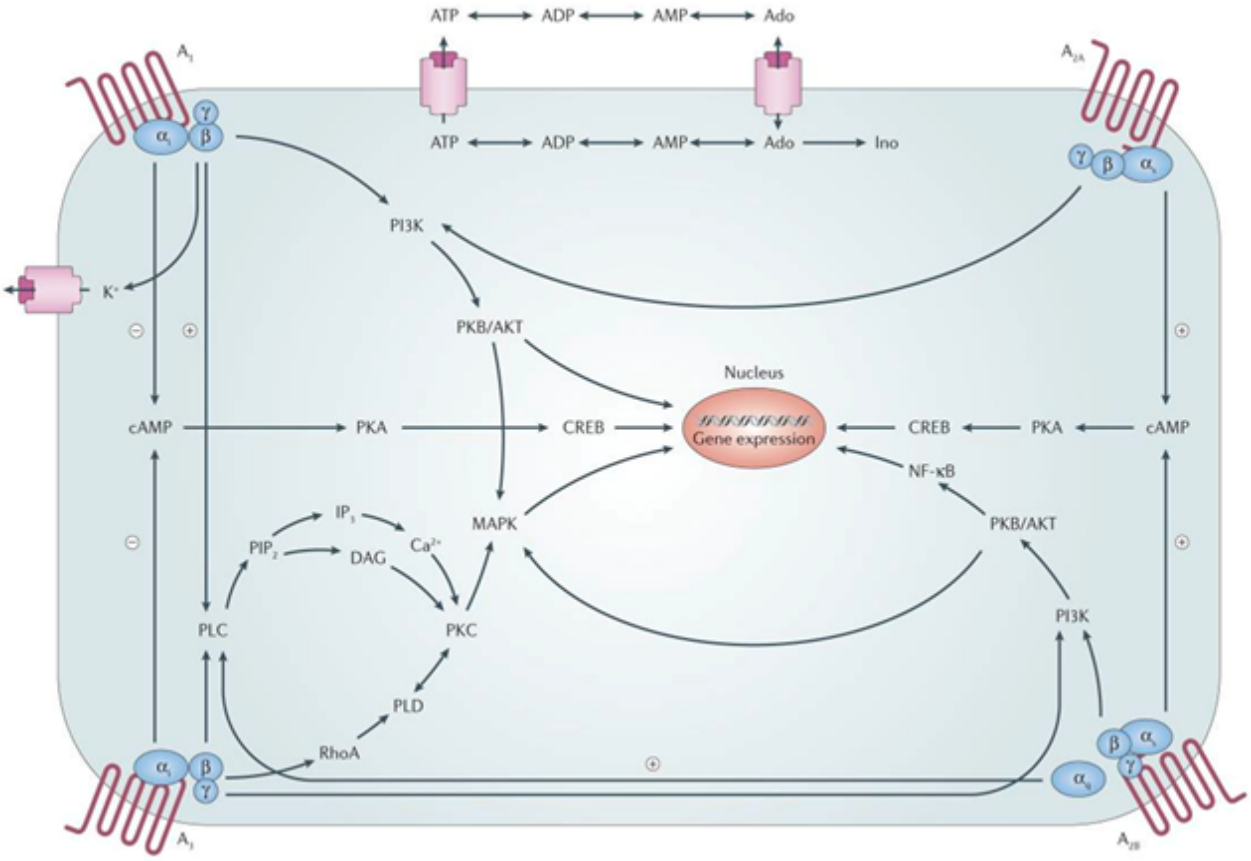

Fig 5: Adenosine receptor signalling pathway. Adenosine initiates its biological effects via four receptor subtypes, each of which has a unique pharmacological profile and effector coupling. The importance of these receptors in the regulation of a great variety of physiological functions makes them promising therapeutic targets in a wide range of conditions. DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphospate; PI3K phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLD, phospholipase; NF-kB, nuclear factor-kB (Jacobson and Gao, 2006).

Because of ubiquitous distribution of ARs throughout the organism, adenosine pathway has been implicated in several biological functions including cardiac rhythm and circulation, angiogenesis, synaptic plasticity, inflammatory diseases and neurodegenerative disorders (de Mendonca and Ribeiro, 1997; Fredholm, 2007; Hasko et al., 2008; Liu et al., 2010). Adenosine is also reported to provide a potent and long-lasting pain relief in both preclinical animal models and human subject studies (Hayashida et al., 2005; Zylka, 2011).

A role for adenosine in antinociception was first identified in the 1970s and then elaborated in the 1980s with systemic and spinal administration of those compounds that, at the time, were considered selective agonists. These first studies emphasized the role of A_1 and A_{2A} ARs (the only subtypes known at that time) and since then up to the least 10 years research has been focused on the use of A_1AR or A_{2A}AR agonists for the treatment of pain of different etiologies. It has been demonstrated, in cultured cells and mouse DRG, that sustained activation of A_1AR leads to depletion of phosphatidylinositol 4,5-biphosphate (PIP2) resulting in an inhibition of thermosensation through TRPV1 and a reduction of thermal hyperalgesia and mechanical allodynia induced by inflammation or nerve injury (Sowa et al., 2010). A_{2A}AR agonists were found to enduringly reverse allodynia and hyperalgesia caused by nerve injury in rats (for up to four weeks after a single intrathecal injection) (Loram et al., 2009).

However, targeting adenosine endogenous pathway using A_1AR or A_{2A}AR agonists failed to yield a viable therapeutic approach: although these agonists are in clinical development for neuropathic pain, their use is restricted to local delivery, since a systemic administration would risk cardiovascular side effects from the activation of A_1AR expressed in conducting tissues or A_{2A}AR in vascular smooth muscle (Boison, 2013; Jacobson et al., 2011; Taliani et al., 2010; Zylka, 2011).

1.2.2 A_3 Adenosine Receptor (A_3AR): a novel target for the treatment of neuropathic pain

Although the basic science suggests that selective AR modulators have promise for numerous therapeutic applications, in practice this goal has been elusive. One reason for this is the ubiquity of ARs and the possibility of side effects. In addition, species differences in the affinity of putatively selective ligands complicate preclinical testing in animal models. However, there has been an impetus towards novel clinical targets, in part as a result of the discovery of the A_3AR subtype in the early 1990s and of the elucidation of new roles for adenosine.

The generation of cDNA for A_3AR has allowed the demonstration of the wide expression of this receptor subtype throughout the organism: its mRNA was found expressed in the testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon, and eye (Burnett et al., 2010; Dixon et al., 1996; Linden, 1994; Salvatore et al., 1993) also if marked differences in expression levels do exist within and between species. Looking at the structures involved in nociception and pain, A_3AR expression has been reported in thalamus, hypothalamus and amygdala (Dixon et al., 1996; Yaar et al., 2002) and electrophysiologic and biochemical evidence suggests the presence of A_3AR also in hippocampus (Lopes et al., 2003) and cortex (Brand et al., 2001). The presence of A_3AR in peripheral sensory neurons (Ru et al., 2011) and at motor nerve terminals (Cinalli et al., 2013) was recently demonstrated. At cellular level, A_3AR expression has been observed in microglia and astrocytes, the resident immune cells of the CNS (Gessi et al., 2013; Ohsawa et al., 2012). It is worth nothing that A_3AR has been found expressed in a variety of immune cells and its activation is involved in the physiopathological regulation of inflammatory and immune processes mediated by adenosine (Antonioli et al., 2010; Hasko and Cronstein, 2013). Human eosinophils were the first cells in which A_3AR was detected (Kohno et al., 1996), then followed by neutrophils (Bouma et al., 1997; Corriden et al., 2013), macrophages (McWhinney et al., 1996), monocytes (Broussas et al., 2002), dendritic cells (Fossetta et al., 2003), lymphocytes (Gessi et al., 2004), bone marrow cells and lymph nodes (Bar-Yehuda et al., 2011).

Since from its discovery A_3AR presented a twofold nature: its activation appeared to be protective and harmful, pro and anti-inflammatory, pro and anti-tumoral, depending on the system investigated and the different pathophysiological conditions considered. This was due to the fact that agonists used at that time were not selective, thus they activated also other AR subtypes which mediate pro-inflammatory responses. However, during the last decade of research, several studies have been carried out to clarify the role of A_3AR agonism and antagonism in different pathologies. A_3AR modulation has been demonstrated to be a winning strategy in the treatment of rheumatoid arthritis (Baharav et al., 2005; Ochaion et al., 2006), inflammatory bowel diseases (Antonioli et al., 2010; Guzman et al., 2006), uveitis (Bar-Yehuda et al., 2011), myocardial and skeletal muscle ischemia (Wan et al., 2008; Zheng et al., 2007) and cancer (Cohen et al., 2011; Fishman et al., 2003; Fishman et al., 2001). In addition to its role as a therapeutic target, A_3AR is now recognized also as a biological marker given its overexpression in inflammatory and cancer cells, compared with low levels found in healthy cells (Fishman et al., 2006; Gessi et al., 2004; Madi et al., 2004; Morello et al., 2008; Ochaion et al., 2009). An important aspect to underlie is that, in contrast to A_1AR and A_{2A}AR

agonists, the activation of the A_3AR in humans by potent, selective, and orally bioavailable A_3AR agonists is not associated with cardiac or hemodynamic effects (Silverman et al., 2008).

Endogenous adenosine signalling through A_3AR has also been demonstrated to be neuroprotective (Boison et al., 2010; Fishman et al., 2012) and some studies, especially in the last few years, have deeply investigated the role of this receptor in pain, using different animal models. One of the first studies carried out by Yoon and colleagues, has examined the effects of A_3AR agonists in mice, using formalin test (Yoon et al., 2005). In this work, intrathecal delivery of A_3AR agonist (IB-MECA) attenuated the inflammatory component, phase 2 but not phase 1, of the formalin test, demonstrating that A_3AR is responsible for the modulation of nociceptive mechanisms underlying central sensitization. In 2012 Dr Salvemini's group has demonstrated for the first time that activation of the A_3AR reverses established mechano-allodynia in mice which underwent chronic sciatic nerve ligation (CCI) and blocks the development of chemotherapy-induced peripheral neuropathy (CIPN) in rats following the administration of widely used chemotherapeutic agents (bortezomib, oxaliplatin and paclitaxel) with distinct antitumor mechanism of action (Chen et al., 2012). Is noteworthy that the analgesic effects of A_3AR agonists are naloxone insensitive, and thus are not opioid receptor mediated, and are ≥1.6-fold more efficacious than morphine and >5-fold more potent. These findings provided the scientific rationale and pharmacological basis for considering a therapeutic development of A_3AR agonists for the treatment of chronic pain. Other studies conducted by the same group provided new insights in the role of A_3AR modulation in CIPN. In particular, it has been shown that A_3AR agonists block the development of paclitaxel-induced neuropathic pain by inhibiting the activation of spinal NADPH oxidase.

This leads to the blockade of redox-dependent signalling pathways (NFκB and MAPK) and a decrease of glial-associated neuroexcitatory/pro-inflammatory cytokine production (TNF-α and IL-1β). At the same time, A_3AR agonists restores glutamatergic homeostasis by blocking the nitration/inactivation of glutamate transporter GLT-1 and glutamine synthetase (Fig 6A). Treatment with the selective A3AR agonists also increases the formation of the neuroprotective/anti-inflammatory cytokine, IL-10 (Janes et al., 2014). Afterwards, it has been demonstrated that, in CIPN model, A_3AR agonists treatment also prevent astrocytic hyperactivation in the spinal cord, which is responsible for the maintenance of chronic pain induced by chemotherapeutic drugs (Janes et al., 2015).

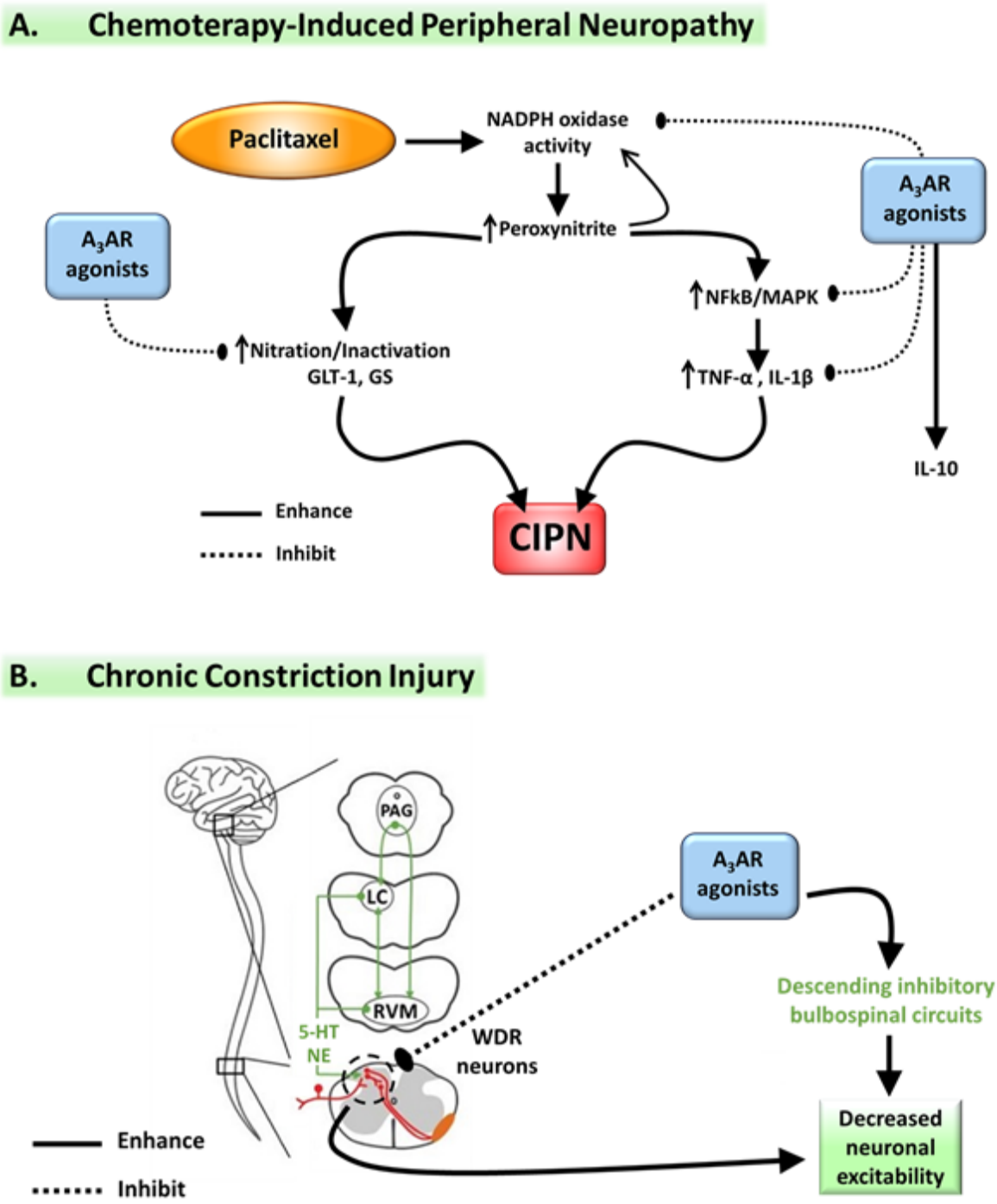

Fig 6 Proposed schematic representation of mechanisms underlying A_3AR agonists' beneficial effects in CIPN (A) and CCI (B) model. A) Chemotherapy (paclitaxel)-induced neuropathic pain is associated with increased NADPH oxidase activity within the spinal cord contributing to enhanced peroxynitrite (PN) production. PN induces the nitration/inactivation of glutamate transporter 1 (GLT-1) and glutamine synthetase (GS) and the activation of redox-dependent signaling pathways (NFκB and MAPK) leading to a surge in glial-associated pro-inflammatory cytokine production (TNF-α and IL-1β). Treatment with the selective A_3AR agonist not only inhibits paclitaxel-induced pain and the associated spinal events, it also increases the formation of the neuroprotective/anti-inflammatory cytokine, IL-10. (Janes et al., 2014) **B**) Stimulation of Periacqueductal Gray (PAG) by different brain regions (not shown) initiate descending pain inhibition. PAG communicates both directly with the Rostro Ventral Medulla (RVM) and through the Locus Coreuleus (LC) to send descending noradrenergic (NE) and serotoninergic (5-HT) inhibitory projections to the spinal cord (Ossipov et al., 2014). A_3AR activation in the RVM engages bulbospinal inhibitory circuits to suppress spinal nociception. At the same time A_3AR stimulation at spinal cord level directly inhibits the excitability of spinal wide dynamic range (WDR) neurons (Little et al., 2015).

Significant progress has been achieved also in understanding the contribution of A_3AR stimulation in alleviating CCI-induced neuropathic pain. Administration of A_3AR agonists following sciatic nerve ligation totally abrogate mechano-allodynia without promoting analgesic tolerance or inherent reward. Further examination revealed that A_3AR activation reduced spinal cord pain processing by decreasing the excitability of spinal wide dynamic range neurons and producing supraspinal inhibition of spinal nociception through activation of serotonergic and noradrenergic bulbospinal circuits (Little et al., 2015) (Fig 6B).

Using the same animal model, it has been also demonstrated that the analgesic effects of A_3AR agonists are due also to the modulation of GABA activity (Ford et al., 2015). The deregulation of GABA signaling in pathophysiological pain states is well established: GABA signaling can be hampered by a reduction in extracellular GABA synthesis by GAD65 and enhanced extracellular GABA reuptake via the GABA transporter, GAT-1. In neuropathic pain, GABAAR-mediated signaling can be further disrupted by the loss of the KCC2 chloride anion gradient. Ford and colleagues have shown how A_3AR selective agonists restore the function of proteins involved in the regulation of GABA bioavailability as well as KCC2 function in CCI animals.

It has been reported that peripheral nerve injury activated also convergent nociceptive inputs from uninjured afferents in the spinal dorsal horn and that microglial activation induced such anomalous inputs (Terayama et al., 2015; Yamamoto et al., 2015). In a recent work, it has been demonstrated that A_3AR selective agonists are able to suppress enhanced microglial activation and thus anomalous convergence of nociceptive inputs in the spinal dorsal horn attenuating neuropathic pain states (Terayama et al., 2018). Engaging the A_3AR mechanism did not alter nociceptive thresholds in non-neuropathy animals (Little et al., 2015).

Although relevant findings have already been reported (Ford et al., 2015; Wahlman et al., 2018; Yan et al., 2016), molecular mechanisms underlying A_3AR agonism remain still largely unexplored. Contrary to the very first studies, which reported a pro-nociceptive role (in large part due to the use of nonselective agonists), is now widely proved that targeting A_3AR induce a robust anti-inflammatory and long lasting antinociceptive effects (Janes et al., 2016).

Thus, A_3 receptor stimulation appears to be a safe and successful strategy for exploiting the potent analgesic action of adenosine to provide a breakthrough non-opioid treatment for patients suffering from chronic pain.

1.2.2.1 A_3AR highly selective agonists

The development of potent and selective synthetic agonists and antagonists of ARs has been the subject of medicinal chemistry research for more than three decades. As mentioned above, A_1 and A_{2a} receptor agonists resulted to have a restricted therapeutic use, whereas there has been a growing interest in the research of novel A_3AR agonists, also due to their beneficial effects against chronic neuropathic pain.

The relationship between the structure of adenosine and its biological activity on the A3 receptor has been extensively explored, and modifications of the adenosine structure have been initially focused on the adenine C_2 and N^6 positions and on the ribose moiety.

Adenosine derivatives bearing an N^6-(3-iodobenzyl) group reported to be 2-fold more selective for A_3 vs A_1 or A_{2a} receptors. The introduction of a 5'-methyluronamide modification in combination with N^6-substitution has generated N^6-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine (**IB-MECA** or CF101) (Fig 7b), a compound which is 50-fold more selective for A_3 vs either A_1 or A_{2a} receptor (Li et al., 1998; Olah et al., 1994; van Galen et al., 1994). Later on, the effects of 2-substituition in combination with modification at N^6- and 5'- position were explored. 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-Nmethyluronamide (**Cl-IB-MECA**) was the first potent and highly selective A_3AR agonist reported (Kim et al., 1994). The introduction of a Cl was observed to incredibly enhance the selectivity for A_3AR subtype: this compound is 2500- and 1400-fold more selective for A_3 vs A_1 and A_{2a} receptors respectively. These prototypical agonists are advancing to phase II and III clinical trials for psoriasis, rheumatoid arthritis and cancer (David et al., 2016; Stemmer et al., 2013). The replacement of the flexible tetrahydrofuryl ring with a conformationally constrained bicyclo[3.1.0]hexane (methanocarba) ring system, which enforces a North (N)-envelope conformation, provided an highly optimized structures for specific A_3AR recognition (Jacobson et al., 2000) (Fig 7c). This modified compound, as well as IB-MECA and Cl-IB-MECA, were shown to be active in reducing or preventing the development of CCI- and chemotherapy-induced neuropathic pain in mice and rats (Chen et al., 2012). (N)-Methanocarba (bicyclo[3.1.0]hexane)-adenosine derivatives were subsequently probed for sites of charged sulfonate substitution, to increase the solubility of these compounds, an important quality for good pharmacological tools (Paoletta et al., 2013) (Fig 7d). A negatively charged sulfonate substitution of small molecules, indeed, is one means of excluding diffusion across biological membranes such as the blood brain barrier. Such potent and

selective A_3AR agonists would be useful in delineating in vivo effects in the CNS from those in the periphery, depending on the route of administration.

During the last 5 years, research focused on further expand the family of A_3AR agonists for the treatment of chronic neuropathic pain. In particular, several modifications of ring structures appended to the 2-ethynyl group were studied, such as heterocyclic groups, aryl groups and cycloalkyl groups (Tosh et al., 2014). In vivo activities of these compounds were compared in correlation with their structure and some preferred candidates have been identified.

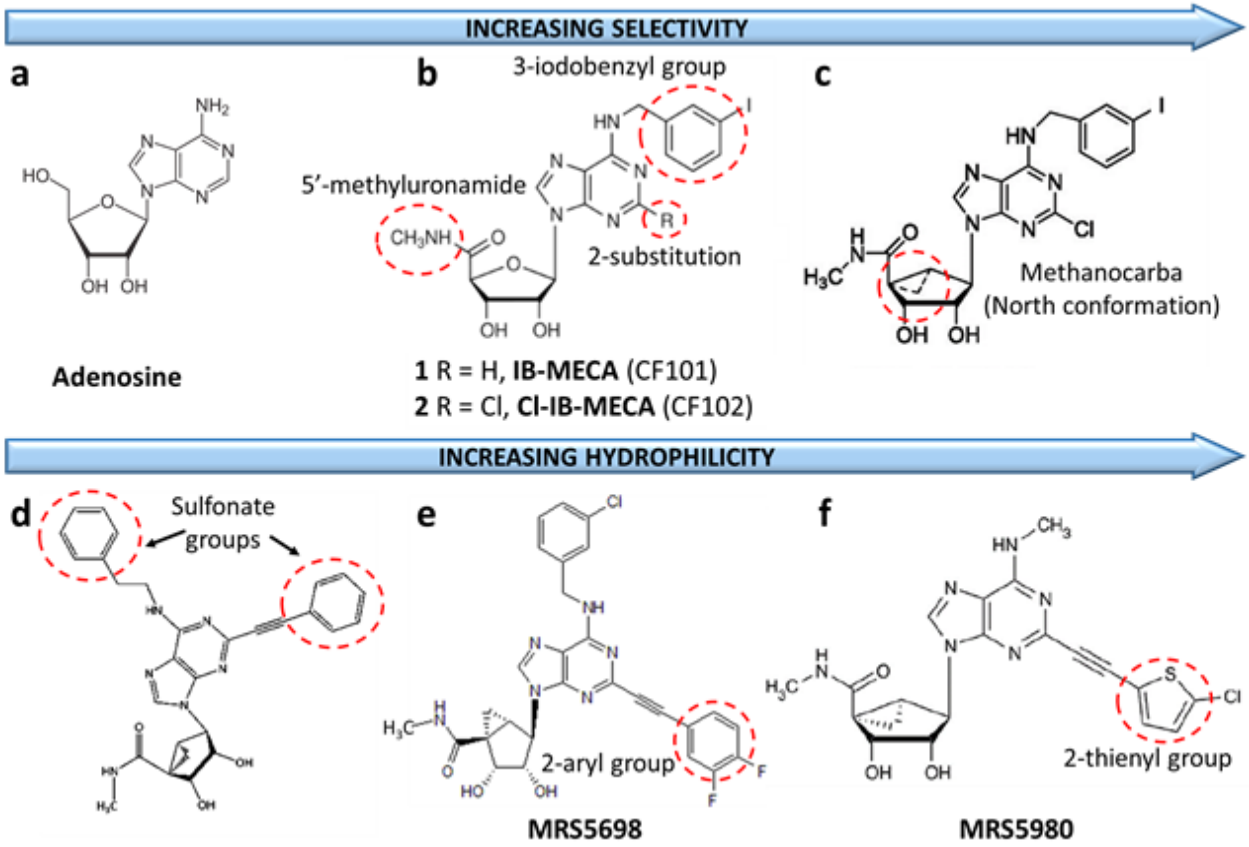

Fig 7. Evolution of synthetic highly selective A_3AR agonists. Chemical structures of typical adenosine derivatives as A_3AR agonists [Figure adapted from (Paoletta et al., 2013; Tosh et al., 2015a; Tosh et al., 2015b).]

Interestingly, it has been found that 2-(arylethynyl)adenine compounds were nanomolar full agonists of A_3AR and, in particular, the highly selective (1S,2R,3S,4R,5S)-4-(6-((3-chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (**MRS5698**) first became an important pharmacological tool for defining A_3AR effects in a chronic neuropathic pain model (Tosh et al., 2012; Tosh et al., 2015b) (Fig 7e). Although the molecular weight is higher than generally preferred, MRS5698 displays good efficacy and

bioavailability. Similarly, it has been demonstrated that also 2-thienyl derivatives are some of the most efficacious analogues in vivo, and, among them, (1S,2R,3S,4R,5S)-4-(2-((5-Chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (**MRS5980**) appeared to be the leading candidate molecule for *in vivo* studies on chronic neuropathic pain (Fig 7f). It has a favourable balance of high and prolonged efficacy (>3h), predicted in vivo stability, very few off-target interactions and high hydro solubility. Moreover, physiochemical parameters of this compound were optimized to further increase its bioavailability. The ED_{50} value for MRS5980 at its peak effect following oral administration is 0.34 mg/kg (0.73 mol/kg; n=5) (Tosh et al., 2015a). Recently, more intensive *in vivo* and *in vitro* analysis have been conducted to better characterize MRS5980 pharmacological profile. Drug efficiency, toxicity, and metabolic elimination are the major properties determining whether a drug candidate can successfully enter clinical trials (Fang and Gonzalez, 2014). For this reason, Fang and colleagues have used metabolomics to evaluate the MRS5980-body metabolism and interactions, including the metabolic elimination of MRS5980, electrophilic reactivity and influence on lipid profiles of MRS5980 in the organism (Fang et al., 2015).

A_3AR agonists are more potent than currently used analgesics (gabapentin, amitriptyline, morphine) (Chen et al., 2012) and, so far, showed an excellent safety profile and efficacy for the treatment of inflammatory, ophthalmic and liver diseases. The novel concept of using these agonists, such as MRS5980, also for treating chronic neuropathic pain is very attractive. However, additional preclinical characterization is needed prior to a proposed clinical trial.

1.3 PROKINETICIN SYSTEM: A MULTIFUNCTIONAL FAMILY OF CHEMOKINES

1.3.1 Cytokines and chemokines involvement in inflammation and pain perception: focusing on prokineticin system

Immune and nervous system have evolved to provide regulation of physiological homeostasis and to protect against threats (Gonzalez et al., 2014). The role of immune system is to defend the organism against infections and injuries, whereas, the nervous system integrates all the biological functions and provides nearly instantaneous homeostatic control mechanisms by releasing neurotransmitters and other regulatory molecules (Chavan et al., 2017). Both systems have the capability to recall earlier challenges and events, mounting memory responses that anticipate and efficiently adapt to ever changing conditions. Nervous and immune cell functions rely on cell-to-cell contacts and on soluble molecules that act on proximal or distant target cells. These communication molecules include neuropeptides, neurotrophins, cytokines and chemokines (Ordovas-Montanes et al., 2015). Cytokines are small (5-30 kDa) active molecules produced by a broad range of cells including macrophages, lymphocytes, mast cells as well as endothelial cells, fibroblasts and various stromal cells. Cytokines are pleiotrophic secreted proteins that were originally characterized as immune modulators but have subsequently been found to mediate a diverse array of functions also in non-immune tissues, including the nervous system. The typical feature of most cytokines is a low or no constitutive production and a transient expression (they exert their effects at concentrations within the pico- nanomolar range) following the inducing stimuli. The term "cytokine" is derived from a combination of two Greek words: "cyto", which means "cell" and "kinos" meaning "movement", due to the observation of their capability to induce the migration of immune cells towards the sites of inflammation, infection and trauma. Cytokines, more in general, are involved in cell-to-cell communication: through both autocrine and paracrine signalling, they actually regulate multiple biological functions such as embryonic development, hematopoiesis, cell growth, differentiation and aging (Foster, 2001). Cytokines operate within a complex network and can act synergistically or antagonistically (Fig 8). Based on the functional profile of an immune response cytokines' production is broadly orchestrated by T helper 1 cells (Th1) which generally mediate a pro-inflammatory cellular immune response, and T helper 2 cells (Th2) which, instead, enhance and anti-inflammatory and humoral immune reaction. Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), prime a Th1 response, enhancing the elimination of

intracellular pathogens. On the other hand, the anti-inflammatory cytokines such as Interleukin-4 (IL-4) and Interleukin-10 (IL-10) drive the Th2 response, enabling phagocytosis of extracellular pathogens and debris, tissue repair and dampening the synthesis of pro-inflammatory cytokines (Kronfol and Remick, 2000).

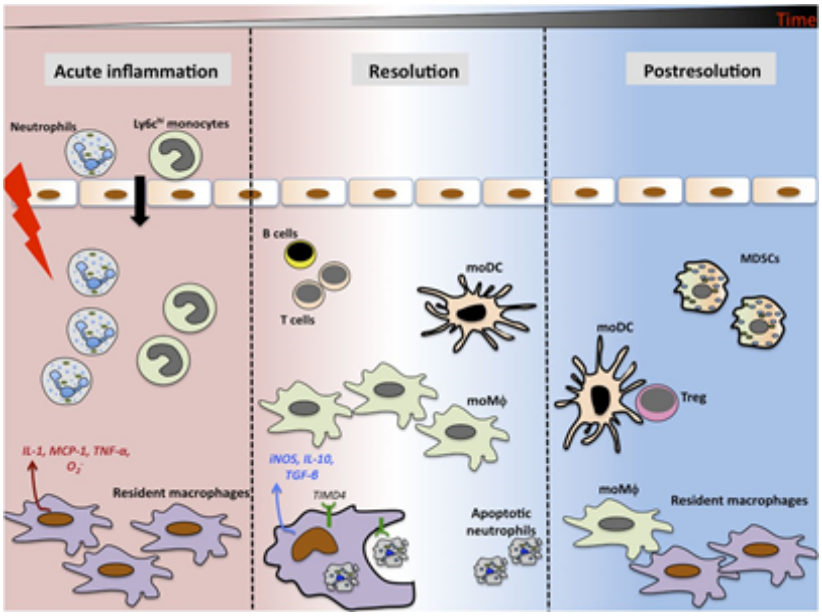

Fig 8. The overall effect of an inflammatory response is dictated by the balance between pro- and anti-inflammatory mediators [source (Bagaitkar, 2014)].

Presently, more than 200 cytokines are recognized. As we said, this class include interferons, interleukins, lymphokines, tumour necrosis factors and chemokines. Chemokines (*Chemo*attractant cyto*kines*), originally identified as regulators of peripheral immune cell trafficking, represent a unique class of neuromodulators that can regulate phenomena as diverse as development, neuroinflammation and synaptic transmission. Chemokines exert their biological effects through cell-surface receptors that belong to G protein–coupled receptors (GPCRs) family. The structure of these receptors is a single polypeptide chains of about 350 residues spanning 7 times the membrane, three intracellular and three extracellular hydrophilic loops, a short amino-terminal (N-terminal) extracellular domain and a serine/threonine-rich intracellular carboxyl-terminal (C-terminal) domain, important for receptor regulation. In the first two extracellular loops are present 2 of 4 conserved cysteine residues that allow the formation of the first disulphide bound required for the definition of the molecular structure, whereas, the second one is due to the bound between the N-terminal domain and the third extracellular loop structure (Bonecchi et al., 2009). Chemokine receptors activate various signalling

pathways, such as the Mitogen-Activated Protein Kinase (MAPK) pathway, the Phospholipase C (PLC) pathway resulting in Ca^{2+} influx, and the phosphatidyl inositol-3 kinase (PI3K) pathway (Bajetto et al., 2002; Cartier et al., 2005), leading to varied functional outcomes, including adhesion, polarization and chemotaxis.

About twenty years ago, for the first time by Dr Melchiorri and Dr Negri group, a small peptide of 77 amino acids was isolated from skin secretions of *Bombina Variegata* frog and called Bv8, to indicate its origin and its molecular weight of 8 KDa. Homologues of this protein were founded in spiders (atracotoxin-Hvf17) and snakes (Mamba Intestinal Toxin-1, MIT1) (Mollay et al., 1999). In particular, the amino acid sequence of Bv8 is very similar to MIT-1 since they share 58% of sequence identity (Negri et al., 2002). This observation suggested that similar proteins could be found also in other species, including mammals. Analysing cDNA libraries using functional cloning, it was possible to identify homologues of Bv8 in mice (Wechselberger et al., 1999) and in rats (Masuda et al., 2002). Later on, Li and colleagues identified two genomic sequences in human which gave origin to two proteins similar to Bv8, called Prokineticin 1 (PROK1) and Prokineticin 2 (PROK2) referring to their ability to contract guinea pig ileum in vitro (Li et al., 2001). During the same period, Ferrara and colleagues identified a protein able to induce proliferation, migration and fenestration of endothelial cells in testis ovary and adrenal glands (LeCouter and Ferrara, 2003; LeCouter et al., 2003a). This protein was called endocrine-gland-derived vascular endothelial growth factor (EG-VEGF) since its effects were similar to the ones induced by VEGF (LeCouter et al., 2001). Actually, EG-VEGF and PROK1 are the same protein.

All these proteins share some structural characteristics (Fig 9):

- Identical amino-terminal (N-terminal) sequence important for the biological activity and receptor recognition (alanine, valine, isoleucine, threonine, glycine and alanine, AVITGA sequence), for this reason, they are also named ‘AVIT proteins’ (Bullock et al., 2004; Kaser et al., 2003; Negri et al., 2005);
- 10 cysteine residues with identical spacing that define a five disulphide bridges, motif called a colipase fold, that confer to the molecule a compact three-dimensional conformation and high protection from enzymatic degradation (Kaser et al., 2003);
- A tryptophan (W) residue in position 24, very important for receptor binding.

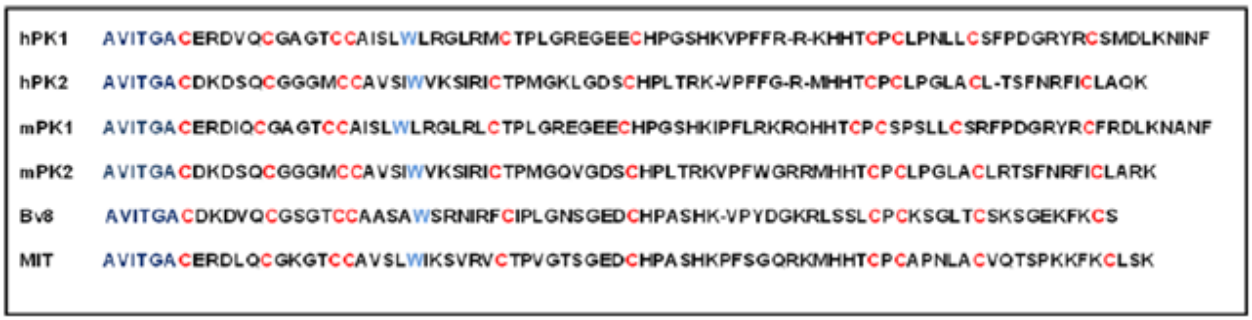

Fig 9. Amino acid sequences of human and mouse PRO Ks and their homologues from frog (Bv8) and snake (MIT). AVITGA - dark blue; cysteine (C) - red; tryptophan (W) - light blue.

The amount of data obtained in these first 20 years of research has allowed to classify prokineticins as chemokines, despite the fact that phylogenetic studies revealed higher similarity with defensins (Monnier and Samson, 2008). Indeed, together with chemokines, Prokineticins are small (8-10 KDa), highly basic, secreted peptides which bind sulphate proteoglycans; they contain a high number of cysteine residues and are potent chemoattractant (Monnier and Samson, 2008). In addition to the initial description of prokineticins as molecules able to induce smooth muscle contractility in the gastrointestinal tract, a number of studies have examined other functions of these proteins in mammals, such as angiogenesis, neurogenesis, circadian rhythm metabolism, haematopoiesis, immune response, reproduction, pain perception (Fig 10). Moreover, the disruption of prokineticin system has been implicated in several pathological conditions, including cancer (Shojaei et al., 2007), immunological response (Monnier and Samson, 2008), mood disorder (anxiety/depression) (Kishi et al., 2009; Li et al., 2009), cardiomyopathy (Attramadal, 2009) and persistent pain (Negri et al., 2009).

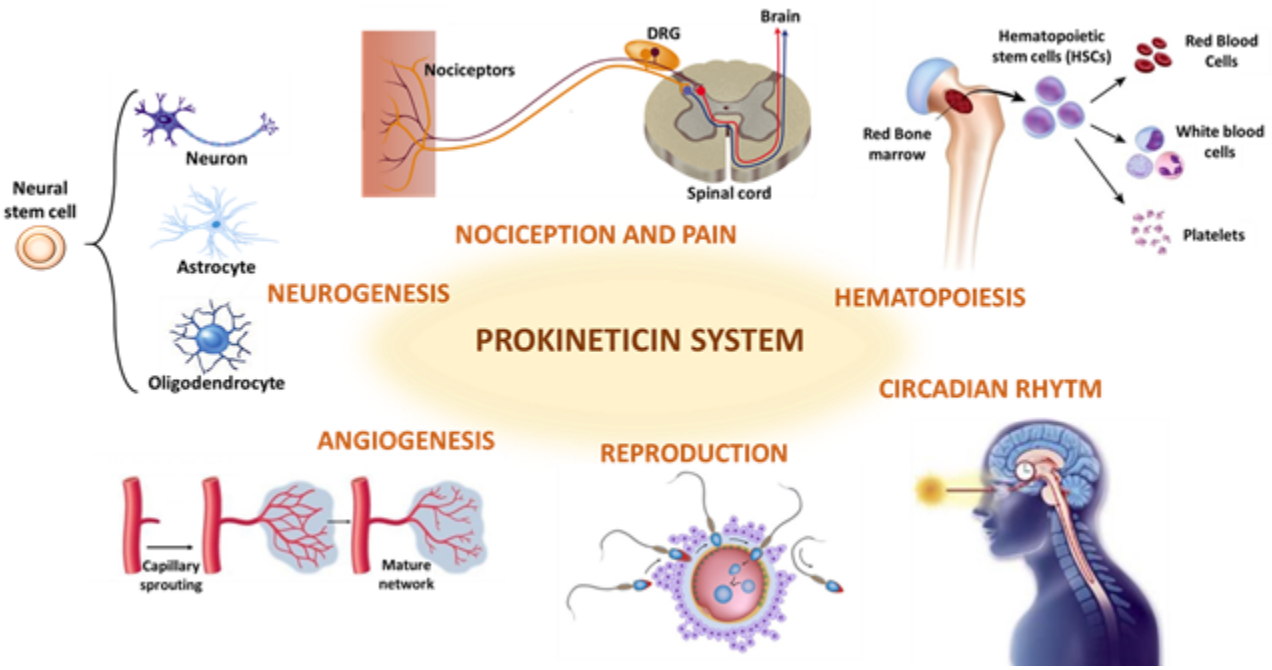

Fig 10. Prokineticin system is involved in several biological functions.

The prokineticin system plays a key role in coordinating injury-associated nociceptive events because it can regulate inflammatory responses and can simultaneously act on different elements of nervous system, both at central and at peripheral level (Fig. 11). The activation of prokineticins' receptors can elicit nociception, contributing to nociceptor sensitivity to different stimuli. The first discovery of the hyperalgesic effect of the prokineticins came from the observation that systemic injection of the amphibian Bv8 in rats induces a characteristic biphasic hyperalgesia to tactile and thermal stimuli (Negri et al., 2002). The initial phase of hyperalgesia is caused by a local action on nociceptors, the secondary phase, instead, is attributable to a central action (De Felice et al., 2012; Negri et al., 2006) indicating differences in the contribution of the prokineticin system at central vs. peripheral sites. Prokineticins and their receptors are expressed in regions of the nervous system associated with pain (Negri and Lattanzi, 2012). In peripheral nervous system, it has been shown that in DRGs prokineticins' receptors are expressed together with the transient receptor potential vanilloid 1 (TRPV1) and with the transient receptor potential ankyrin 1 (TRPA1). These co-localizations provide the anatomical basis for a cooperative interaction in nociceptor sensitization through activation of PKCε (Negri et al., 2006; Vellani et al., 2006). In rat primary sensory neurons PROK2 also enhances proton-gate current, suppresses GABA-activated current and sensitizes P2X receptors, via PKC signal pathway (Qiu et al., 2012; Ren et al., 2015; Xiong et al., 2010). Evidence that the prokineticin system is directly involved in setting the pain threshold comes from studies in mice lacking specific prokineticins' genes: PKR1, PKR2 and PROK2 knock out animals display higher thermal,

mechanical and tactile pain threshold compared to wild type mice (Hu et al., 2006; Negri and Lattanzi, 2012; Negri et al., 2006). At central level in the spinal cord, the highest density of prokineticins' receptors has been found within the dorsal horns indicating that these receptors are involved in central transmission of the nociceptive signal (Negri and Lattanzi, 2012). Moreover, prokineticins' receptors are present on astrocytes also containing PROK2 (Guida et al., 2015; Lattanzi et al., 2015; Maftei et al., 2014). The prokineticn system also intervenes in modulating central pain mechanism. Intra periacqueductal gray (PAG) injection of amphibian protein Bv8 exerts a pro-nociceptive action by increasing the intrinsic GABA-ergic tone that, in turn, is responsible for the inhibition of PAG anti-nociceptive output neurons impinging on rostral ventromedial medulla neurons (de Novellis et al., 2007; Negri and Lattanzi, 2012). Co-injection with the partial agonist A-24 abolished this central effect of Bv8. Peripheral administration of A-24, besides antagonizing the Bv8-induced hyperalgesia, increased the opioid content in hypothalamus and midbrain of mice (Lattanzi et al., 2012).

Lymphoid organs, circulating leukocytes and haematopoietic cells, synoviocytes and dendritic cells constitutively express moderate levels of PROK2. Inflammatory stimuli activate the release, from fibroblast and endothelial cells, of granulocyte colony-stimulating factor (G-CSF), a major inducer of PROK2 which is overexpressed in inflamed tissues and in animal and human neoplastic tissue, predominantly in infiltrating neutrophils from which it may be secreted. PROK2, in turn, regulates angiogenesis and vessel permeability, activates macrophages and modulates immune responses through prokineticins' receptors expressed on capillary endothelial cells and on leucocytes (Dorsch et al., 2005; Franchi et al., 2008; LeCouter et al., 2003b; Martucci et al., 2006; Qu et al., 2012; Shojaei et al., 2009). In an animal model of inflammation produced by Complete Freund's Adjuvant (CFA) injection into the paw of rats or mice, PROK2 mRNA (which is quite undetectable in healthy paws) dramatically increases in the skin, associated with infiltrating cells (granulocytes and macrophages) and temporally correlates with pain and other traits of inflammation as oedema. 24h after the injection, PROK2 is significantly increased also in the DRGs ipsilateral to the paw injected with CFA (Giannini et al., 2009; Negri and Lattanzi, 2011). Granulocyte released PROK2 modulates acute inflammatory pain directly acting on nociceptors and, in turn, exerts chemotactic activities, induces a proinflammatory macrophage phenotype and skews the Th1/Th2 balance to Th1 (Franchi et al., 2008; Martucci et al., 2006). Hence, besides the direct activation of the nociceptors by PROK2, other cytokines/chemokines, induced by PROK2, contribute to keep pain in chronic inflammation. There are now various reports of prokineticin system dysregulation in inflammatory diseases, for example in gut inflammation (Watson et al., 2012), in testis inflammation (Chen et al., 2016), in a mouse model of multiple sclerosis, the autoimmune encephalomyelitis (Abou-Hamdan et al., 2015), in a mouse model of the human rheumatoid arthritis, Type II collagen induced arthritis (Ito et al., 2016)

(Lattanzi and Cuzzocrea, in preparation). In these mice PROK2 gene expression was significantly elevated in the joints and correlated with the severity of the arthritis. Repeated treatment with prokineticins' receptors antagonists before the onset of arthritis resulted in significantly lower arthritis scores, histological damage and pain, getting along with reduced expression of PROK2 in the synovia.

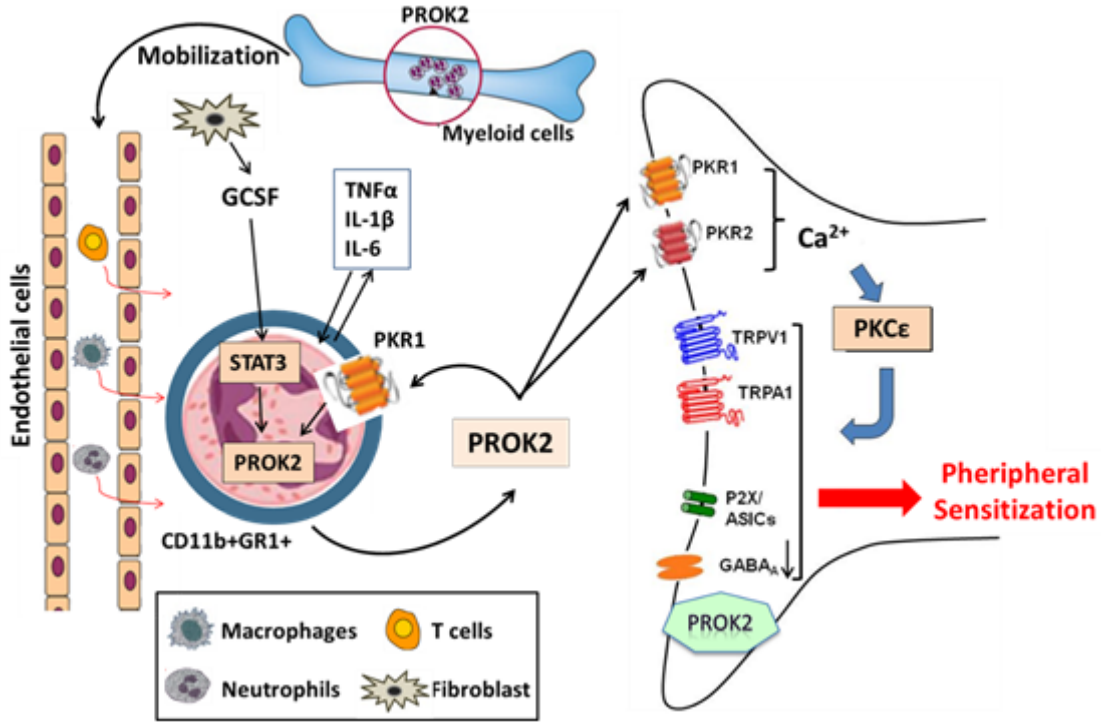

Fig 11. PROK2 induces inflammation and peripheral sensitization. Bone marrow and circulating leukocytes constitutively express moderate levels of PROK2. Inflammatory stimuli activate the release, from fibroblast and endothelial cells, of G-CSF which, through STAT3 activation, contributes to the recruitment of granulocytes and induces PROK2 overexpression in neutrophils infiltrating the inflamed tissues. The released PROK2 regulates angiogenesis and vessel permeability, activates macrophages and modulates immune responses acting on PKRs expressed by capillary endothelial cells and leucocytes. Moreover, by activating PKR1 and PKR2 on primary afferent neurons, PROK2 modulates pain perception; and by activating the PKRs on nociceptor neurons PROK2 induces Ca2+ mobilization, PKCε translocation, modulation of transducers as TRPV1, TRPA1, ASIC channels, P2X channels and reduction of GABAa efficiency [source (Negri and Maftei, 2018)].

1.3.2 Role of prokineticins in neuropathic pain

Peripheral nerve injury (CCI or SNI) induces the overexpression of PROK2 and its receptor, PKR2, in the sciatic nerve, in lumbar DRGs and in lumbar portion of the spinal cord (Lattanzi et al., 2015; Maftei et al., 2014). Analysing mRNA expression, our group has found out that PROK2 and PKR2 transcripts up-regulation starts 3 days after injury in the sciatic nerve and then it moves towards the central nervous system, becoming significant in the spinal cord after 10 days. PROK2 protein, normally absent in healthy sciatic nerve, becomes detectable following injury in some axons and it is

mainly associated with activated Schwann cells and infiltrating macrophages. Immunofluorescence staining of sciatic nerve proximal to the lesion show a dramatic increase also of PKR2 signal. The release of PROK2 in the nerve contributes to neuroinflammation (Lattanzi et al., 2015). 6 days after injury, both mRNA and protein levels of PROK2 are significantly increased also in the DRG neurons and satellite cells and in activated astrocytes within the spinal cord, but not in microglia. In DRGs PKR2 immunoreactivity, staining the whole cell body, was evident in many neurons one week after surgery. Moreover, at spinal cord level, the increased immunoreactivity of PROK2 associated with synaptophysin (a presynaptic marker) indicates that PROK2 may be transported to the central endings of nociceptor and then released. Eventually, PROK2 released in the spinal cord activates PKR2 which is constitutively localized on the projection neurons and it is up-regulated after nerve injury (Maftei et al., 2014). This contributes to spinal glia activation and to the aberrant excitability of the dorsal horn which generates allodynia and hyperalgesia, prominent symptoms of neuropathic pain. It is to point out that nerve injury induces overexpression also of the receptor PKR1, but only in the sciatic nerve, reaching a maximal expression 10 days post-surgery.

Selective antagonists are extremely useful tool to better characterize the impact of modulations of a specific system. Balboni and colleagues have synthesized and developed several nonpeptidic prokineticin antagonists (Balboni et al., 2008; Lattanzi et al., 2014), among which the lead compound is PC1. This triazine compound mimics the structural features required for prokineticins' receptor binding and experiments conducted by our group demonstrate that PC1 acts as a preferentially PKR1 ligand. Treatment with prokineticin antagonists is highly efficacious in controlling neuropathic pain. A single acute subcutaneous or intraperitoneal injection of PC1 rapidly reduced established pain suggesting a direct action on PKRs expressed by nociceptors, whose blockade decreases spinal neurons sensitization (Guida et al., 2015) and the transmission of painful stimuli. Therapeutic treatment with PC1 (s.c., 1 week) alleviated established thermal hyperalgesia and allodynia, reduced the injury-induced overexpression of PROK2, significantly blunted nerve injury-induced microgliosis and astrocyte activation in the spinal cord and restored the physiological levels of proinflammatory and anti-inflammatory cytokines in periphery and in spinal cord (Maftei et al., 2014). Chronic administration of PC1, starting from the day of injury, blocks the onset of mechano-allodynia and thermal hyperalgesia and delays the reappearance of painful symptoms 2-3 days after treatment suspension, suggesting that blocking PROK2 signalling could induce long lasting changes in neuronal circuits and/or in neuroinflammatory phenomena involved (Guida et al., 2015). PC1 treatment also normalizes the nerve injury increased permeability of the blood-spinal cord barrier (BSCB) (Guida et al., 2015; Maftei et al., 2014) demonstrating the possible involvement of the prokineticin system

in the regulation of the neuroinflammatory phenomena leading the infiltration of the peripheral immune cells into the spinal cord.

1.3.3. Prokineticin receptors: structure, distribution and signalling pathways

Prokineticins exert their biological functions through the activation of two closely related G-protein coupled Receptors (GPCRs): Prokineticin Receptor 1 (PKR1) and Prokineticin Receptor 2 (PKR2) (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). Although human and mouse genes encoding for prokineticin receptors are on two different chromosomes (human: PKR1 gene: 2p13.3; PKR2 gene: 20p13; mouse: chromosome 6 and 2 respectively), the sequences of both receptors are remarkably conserved, displaying more than 85% identity and both of them are about 80% identical to the previously described mouse orphan receptor GPR73 (Parker et al., 2000). Most sequence variation is concentrated in the extracellular N terminal region - which contains a nine-residue insert in PKR1 compared with PKR2 - as well as in the second intracellular loop and in the C terminal tail (Levit et al., 2011) (Fig 12). Western blot analysis performed on human neutrophils has shown that PKR2 is able to form homodimers and, following heterologous expression of PKR2 in Saccharomyces cerevisiae, it has been demonstrated that its dimerization is due to a hydrophobic interaction between transmembrane domains. A PKR1-PKR2 heterodimer has also been observed (Marsango et al., 2011; Sposini et al., 2015).

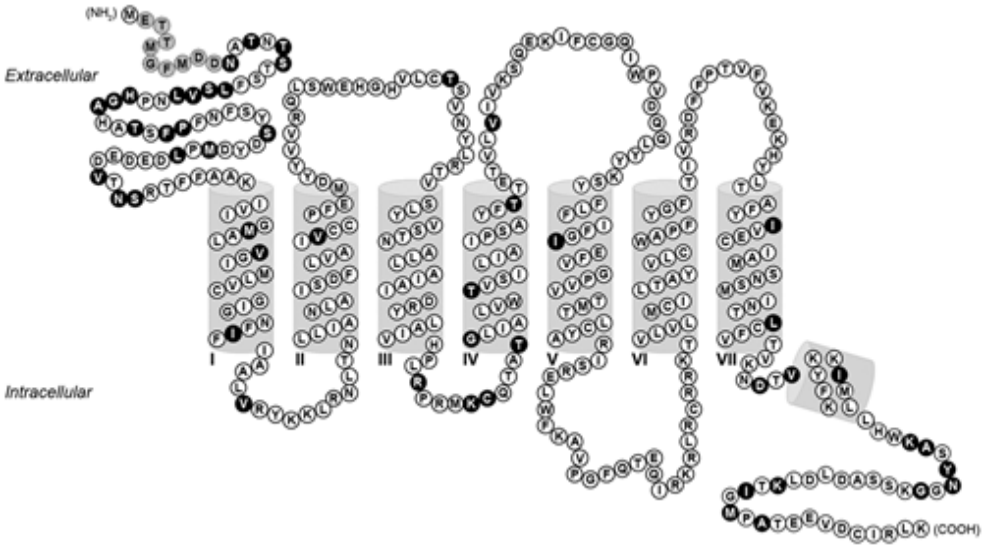

Fig 12. Snake plot of human PKR1. The secondary structure is according to PKR1 protein annotation in the UniProtKB database (entry Q8TCW9). Positions in the PKR1 sequence differing from PKR2 (entry Q8NFJ6) are shaded black. Conserved positions between the two subtypes are shaded white. A nine-residue PKR1-unique insert in the N terminus is shaded gray with dashed lines. The seven transmembrane domains are denoted by roman numerals. Extracellular and intracellular sides of the membrane are labeled, as well as the N terminus (NH2) and C terminus (COOH) ends of the protein (Levit et al., 2011).

Several studies show that both prokineticin receptors are distributed throughout the organism: PKR1 is mainly expressed in peripheral tissues, including endocrine glands and organs of the reproductive system, spleen, gastrointestinal tract, lungs, heart and immune cells (such as neutrophils and macrophages) whereas, in the central nervous system (CNS) PKR2 is more abundantly expressed and PKR1 is present only in discrete brain areas (Cheng et al., 2006; Lin et al., 2002a; Negri et al., 2007; Soga et al., 2002). In particular, PKR2 has been found expressed in the paraventricular nucleus, arcuate nucleus, dorsomedial hypothalamus and subfornical organ which are the brain areas that control ingestive behaviour and the sense of thirst (Gross et al., 1985; Hillebrand et al., 2002; Kalra et al., 1999; Negri et al., 2004). Relevant is also the presence of PKR2 in the suprachiasmatic nucleus where is implicated in the regulation of circadian rhythm (Cheng et al., 2002; Negri et al., 2004) and in several brain areas involved in emotions and mood regulation such as amygdala and hippocampus (Cheng et al., 2006).

Computational (Levit et al., 2011) and genetic (Monnier et al., 2009) analysis indicated the binding sites for the endogenous ligands on the extracellular surface of the receptors in correspondence of the second extracellular loop and those for the small non-peptide PKRs antagonists and agonists, recently identified (Balboni et al., 2008; Gasser et al., 2015), in a pocket located in the upper part of the TM bundles among TMs 3, 4, 5, 6 and 7. Computational analysis suggests an identical TM bundle binding site for PKR1 and PKR2 so that small-molecules are not likely to easily differentiate between the two receptor's subtypes (Lattanzi et al., 2014; Levit et al., 2011). However, it looks like that agonists and antagonists interact with different residues resulting in a totally different area of this pocket. It has been also demonstrated that, following stimulation with selective agonists, PKRs undergo rapid internalization (Gasser et al., 2015). Except for MIT-1, a clearly PKR2-preferring ligand, and PK2β which displays a clear selectivity for PKR1 (Chen et al., 2005), all the other natural prokineticins bind and activate both receptors in nanomolar range, with PROK2 showing a moderately higher affinity than PROK1 for both receptors (Lin et al., 2002a; Soga et al., 2002). The nonmammalian prokineticins (MIT-1 and Bv8) display considerably higher affinity with at least one order of magnitude higher compared with human prokineticins. Bv8, that displays similar affinity for both receptors, behaves as mammalian PROK2 and is a good pharmacological tool to evaluate the prokineticin activities (Negri et al., 2007).

Several studies show that PKR1 and PKR2 are associated with $G\alpha_q$, $G\alpha_{i/o}$ and $G\alpha_s$ proteins. As a consequence of this redundancy, prokineticins' signalling depends on tissue-specific expression of ligands, receptors and associated G proteins. As a result, a huge variety of physiological functions in response to the same ligand stimulation is possible (Fig 13). In PKRs transfected neuronal and specific endothelial cell lines, the activation of PKRs stimulates intracellular calcium mobilization

through $G\alpha_q$ coupling. $G\alpha_q$ activates Phospholipase C-β (PLC-β) with the subsequent formation of inositol 1,4,5-trisphosphate (IP3) (Lin et al., 2002a). The increase of intracellular calcium leads to the activation of calcineurin which subsequently dephosphorylates cytoplasmatic nuclear factor of activated T cells (NFAT). This allows NFAT to migrate to the nucleus and bind to specific promoters inducing genes transcription (Cook et al., 2010). Lin and colleagues have demonstrated in bovine adrenal cortex-derived endothelial (ACE) cells that activation of PKRs induce the phosphorylation of p44/42 mitogen-activated protein kinase (MAPK/ERK) pathway and that this mechanism is pertussis toxin–sensitive, proving that PKRs may also couple to $G\alpha_{i/o}$ protein. (Lin et al., 2002b; Mangmool and Kurose, 2011). All these data are consistent with the effects of prokineticins on smooth muscle contraction and angiogenesis. It has been demonstrated in nociceptors of dorsal root ganglia (DRG) that prokineticin receptors' stimulation increases intracellular concentration of Ca^{2+} by activation of the TRPV1 channels in a dose-dependent manner. In particular, prokineticins' binding to their receptors causes the translocation of protein kinase Cε (PKCε) to the neuronal membrane, inducing an enhancement of inward Ca^{2+} current carried by TRPV1 (Vellani et al., 2006). This cross-talk between TRPV1 and PKRs signalling is crucial for nociception (Hu et al., 2006). Enteric neural crest cells (NCC) are multipotent progenitors which give rise to neurons and glia of the enteric nervous system (ENS) during fetal development. Glial cell line-derived neurotrophic factor (GDNF)/RET receptor tyrosine kinase (Ret) signalling is indispensable for their survival, migration and differentiation. It has been reported that PKR1 is consistently up-regulated by GDNF in enteric NCCs (Ngan et al., 2008; Ruiz-Ferrer et al., 2011) proving that PKRs signalling is also important to maintain proliferation and differentiation of enteric NCCs. It has been measured cAMP accumulation in in human embryonic kidney cells 293 (HEK293) transiently expressing PKR1 or PKR2. The results indicate that prokineticins stimulate cAMP accumulation in PKR-expressing cells specifically. HEK293 cells without PKRs expression did not respond to prokineticins stimulation. The ligand-stimulated cAMP accumulation is significantly increased if the Gs protein is coexpressed with PKRs (Chen et al., 2005). Analysing germline PKR2 variants associated with central hypogonadism, it was possible to demonstrate that the two different PKR2-dependent pathways, inositol phosphate-Ca^{2+} (Gq coupling) and cAMP (Gs coupling) can undergo separate modulation resulting in patients with alterations of distinct intracellular signalling pathways (Libri et al., 2014).

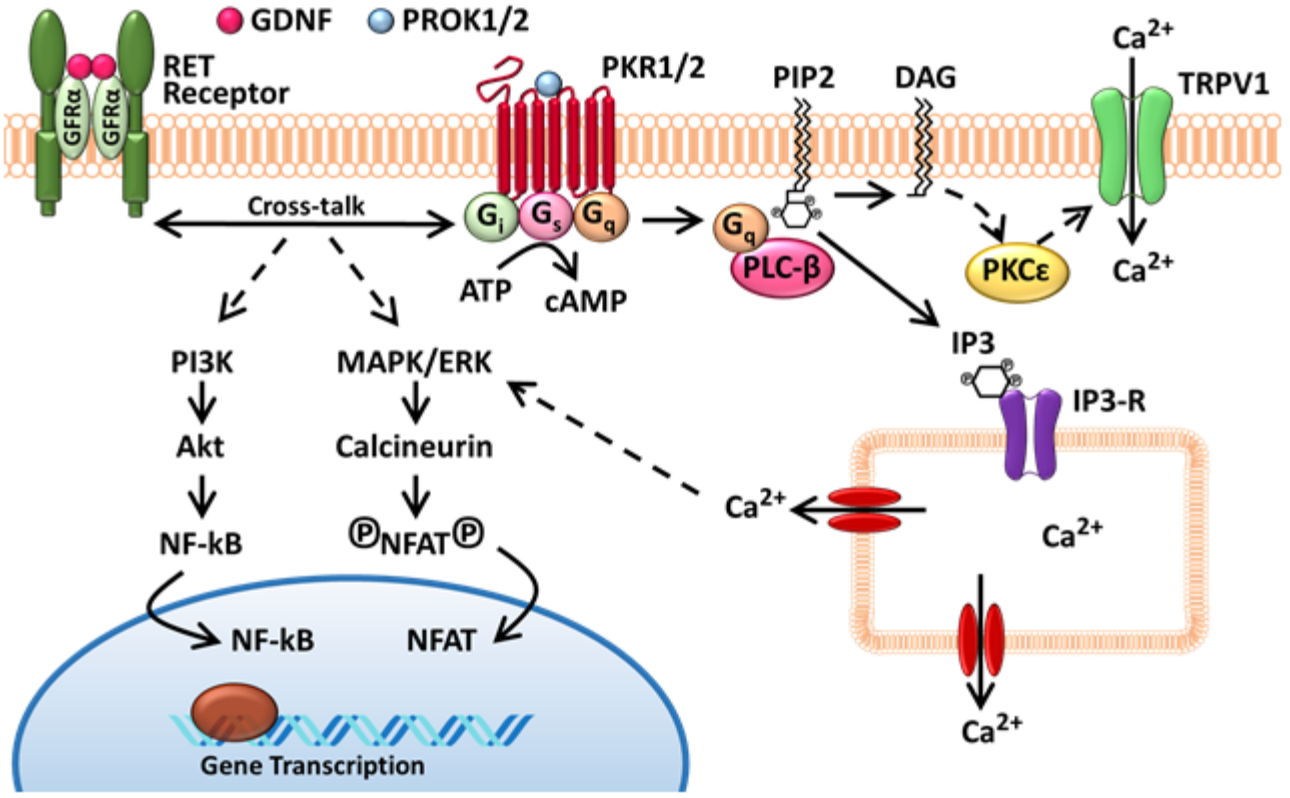

Fig 13. Prokineticin Signalling. PROK1 and PROK2 bind two G-protein coupled receptors (PKR1 and PKR2) which are coupling to Gαi, Gαs, Gαq to activate MAPK/Akt, cAMP accumulation and calcium mobilization, respectively [Figure adapted from (Ngan et al., 2008)].

1.3.3.1 TM4-7: Identification and characterization of Prokineticin Receptor 2 (PKR2) splicing variant

Alternative splicing of precursor messenger RNA (mRNA) is an essential mechanism to increase the complexity of gene expression. Gilbert (Gilbert, 1978) first proposed the concept of alternative splicing in 1978, which is currently the mechanism that accounts for the discrepancy between the number of protein-coding genes (~25,000) in humans and the >90,000 different proteins that are actually generated (1998; 2004).

Constitutive splicing is the process of intron removal and exon ligation of the majority of the exons in the order in which they appear in a gene. During the process of alternative splicing, instead, particular exons of a gene may be included within or excluded from the final, processed mRNA produced from that gene. Consequently, the proteins translated from alternatively spliced mRNAs will contain differences in their amino acid sequence and, more interestingly, in their biological activities (Wang et al., 2015). Alternative splicing has a role in almost every aspect of protein function, including binding between proteins and ligands, nucleic acids or membranes, localization

and enzymatic properties. Taken together, alternative splicing is a central element in gene expression (Kelemen et al., 2013). The recognition by the spliceosome of weaker splicing signals at alternative splice sites is highly dependent on a system of trans-acting proteins that bind to cis-acting sites on the primary transcript itself and, in turn, on cellular context (Chen and Manley, 2009).

In rat and mouse, PKR2 gene (as well as PKR1 gene) is composed by three exons and two introns. The first exon contains a 5' untranslated sequence (5' UTR); the second exon contains a part of the 5' UTR sequence and a region encoding for the first three transmembrane domains (TM1, TM2 and TM3); the third exon encodes for the last four transmembrane domains (TM4, TM5, TM6 and TM7) and for the 3' UTR sequence. The second intron is located at the TM3 border within the common DRY (Asp-Arg-Tyr) sequence (Fig 14). Intriguingly, the conservation of certain exon-intron boundaries and the relatively high sequence homology between rat and mouse suggests that these two receptors are evolutionarily related.

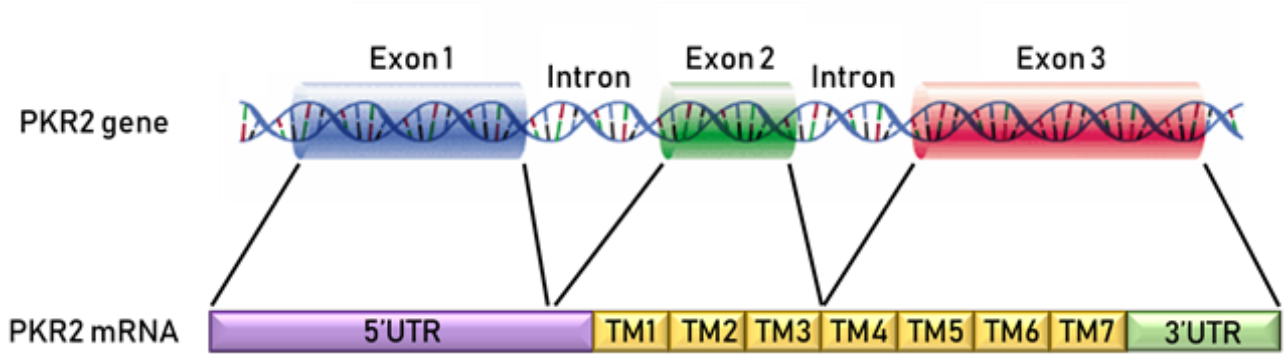

Fig 14. PKR2 gene and mRNA structure. Schematic representation of PKR2 gene structure and the corresponding mature mRNA. Each of the three exons encodes for a specific portion of the final protein.

Several years ago, by computational process (Florea et al., 2005), alternatively spliced mRNA transcript in rat and in mouse for PKR2 gene had already been predicted. However, only recently, it has been demonstrated for the first time the presence of PKR2 splicing variants in vivo in the nervous system (Lattanzi et al., Neuropeptides, submitted). Using designed specific primers, it was possible to identify in rat hippocampus an mRNA encoding for a PKR2 splicing variant, which, lacking the second exon, gives rise to a protein comprising only four transmembrane elements, denominated TM4-7 (Fig 15). Through the expression of this splicing variant in yeast, it was possible to deeply characterize it. Since mutations in GPCRs sequence could impair their stability, it has been firstly demonstrated by fluorescence confocal microscopy that TM4-7 trafficked normally inside the cell and it was found expressed on the plasma membrane like PKR2. Afterwards, using immunoprecipitation assay, it has been showed that TM4-7 is able to interact with PKR2 long form

generating a receptor heterodimer. This last result is in line with previous data showing that interactions between PKR2 protomers involve TM4 and TM5 domains (Sposini et al., 2015).

TM4-7, both as heterodimer and homodimer, shows different functional characteristics in comparison to PKR2 wt dimers: in particular, TM4-7 activation induces Gαq and Gαs, but not Gαi coupling (Lattanzi et al., Neuropeptides, submitted).

Besides the differences in signalling pathways, GPCRs' alternative splice variants often exhibit distinct tissue distribution patterns and drug binding properties compared to the respective wt form. Moreover, alternative spliced forms can be expressed only in particular cell conditions (Markovic and Challiss, 2009; Oladosu et al., 2015). Lattanzi and colleagues have demonstrated, in a murine model of Alzheimer's disease, that TM4-7 transcript was found overexpressed in the hippocampus of rats following intracerebroventricular administration of $A\beta_{1\text{-}42}$ (Lattanzi et al., Neuropeptides, submitted). Interestingly, in the same animals, the increase of PKR2 wt form was minimal compared to the control group, suggesting that the alternative spliced form could be specifically expressed only in inflammatory or pathological conditions. Further studies need to be performed to investigate the role of TM4-7 in distinct inflammatory/neurodegenerative diseases.

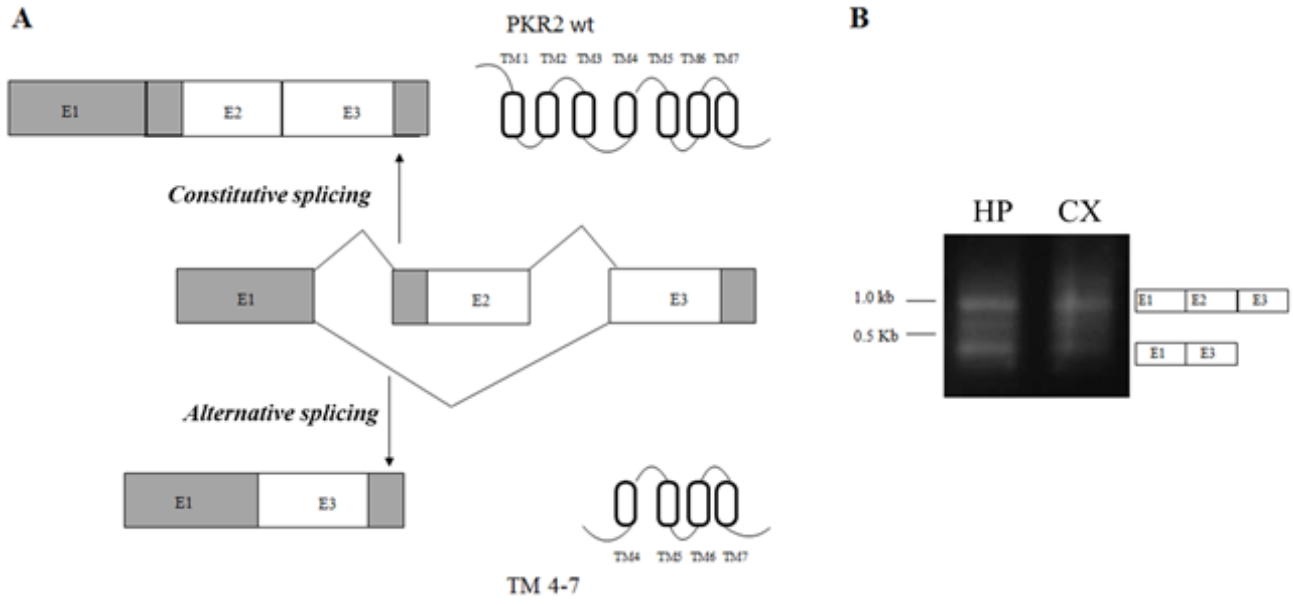

Fig 15. Scheme of alternative splicing of PKR2 gene exons. A) Exon coding sequences are indicated as white bars and untranslated sequences are shown as gray bars. B) Total RNA harvested from hippocampus (HP) and cortex (CX) was analysed for PKR2 expression by RT-PCR. Positions of standard markers are indicated on the left.

2. AIMS OF THE STUDY

Chronic neuropathic pain represents a huge unmet medical need affecting millions of individuals worldwide (Pizzo and Clark, 2012; van Hecke et al., 2014). Opioids have been regarded for millennia as among the most effective drugs for the treatment of acute and persistent pain. However, chronic opioid exposure give rise to a well-known plethora of side effects such as nausea, constipation, tolerance and addiction. Further complications have recently emerged which have the potential to increase the morbidity of patients who are on long-term opioid therapy. They include hypogonadism, osteoporosis, immune suppression, cognitive impairment and hyperalgesia (Raghavan et al., 2011). For these reason novel non-narcotic analgesics are needed.

Animal models are pivotal for understanding the mechanisms of neuropathic pain and the development of effective therapy for its optimal management. A battery of neuropathic pain models has been developed to simulate the clinical pain conditions with diverse etiologies. The present study was conducted using the well characterized chronic constriction injury (CCI) model (Bennett and Xie, 1988) in mice.

In the last decade both adenosine pathway through A3 receptor and prokineticin system have emerged as promising therapeutic targets for the management of neuropathic pain. It is essential to improve our mechanistic understanding of their role in chronic pain states in order to develop novel, safe non-narcotic drugs to present in clinical trials.

Based on these premises, my PhD work was focused mainly on two projects, aimed at the:

I) Investigation of A_3AR agonists' mechanism of action in alleviating neuropathic pain

It has been recently reported that dysregulation of adenosine signalling at the A3 adenosine receptor (A_3AR) subtype contributes to the development of neuropathic pain states. Accordingly, supplementing A_3AR signalling with exogenous application of highly selective A_3AR agonists block and reverse neuropathic pain (Chen et al., 2012; Little et al., 2015; Tosh et al., 2015a; Tosh et al., 2014). These data identified the A_3AR as a target for therapeutic intervention and A_3AR agonists as a novel approach for chronic pain management (Janes et al., 2016). However, the molecular signalling pathways engaged by A_3AR agonism remain elusive.

T cells are essential components of adaptive immunity involved in pain pathology and they actively contribute to homeostasis and repair in the nervous system (Laumet et al., 2018). In response to nerve injury, T cells infiltrate the sciatic nerve, the dorsal root ganglion (DRG) and the spinal cord (Hu and McLachlan, 2002; Lees et al., 2015). Different subsets of T cells are responsible for the production

and the release of pro- or anti-inflammatory cytokines and the balance between them determines the outcome of the inflammatory response (Neurath, 2014). In particular, $CD4^+$ T cells are known to be a prominent source of IL-10 which exerts its action on sensory neurons to reduce pain (Walsh et al., 2015). Increasing the number of $CD4^+$ T cells in neuropathic pain mice attenuates pain responses following CCI surgery, whereas their peripheral depletion resulted in prolonged mechanical pain hypersensitivity (Austin et al., 2012). These findings suggest that $CD4^+$ T cells play a role in endogenous recovery from neuropathy-induced pain. Thus, this T-cell subset may be specifically targeted to alleviate chronic neuropathic pain (Austin et al., 2012; Ji et al., 2016). Previous studies have demonstrated that IL-10 is a strong neuroprotective cytokine which promotes neuronal survival and has remarkable therapeutic effects in neuropathic pain. A single intrathecal administration of IL-10 rapidly reverse mechano-allodynia induced by chronic constriction injury (CCI) improving animals motor functions for few hours (Lee et al., 2013; Shen et al., 2013; Zhou et al., 2009a), whereas viral-mediated delivery of IL-10 after nerve injury has resulted in a more sustained reversal of both mechano-allodynia and thermal hyperalgesia for at least 4 weeks (He et al., 2013; Lau et al., 2012). Other studies have shown that exogenous administration of IL-10 suppresses allodynia in different pain models (Shen et al., 2013) and that inhibition of endogenous IL-10 signalling by intrathecal administration of IL-10 antibody prolonged thermal hyperalgesia in a model of transient inflammatory pain (Willemen et al., 2014). A_3ARs are expressed on the membrane of $CD4^+$ and $CD8^+$ T cells and their expression is increased under pathological settings in correlation with the progression of inflammatory response (Ochaion et al., 2009).

Based on this existing literature, we hypothesized that T cells may be a cellular substrate for A_3AR agonism. To asses this, wild type, $Rag1^{-/-}$, $A_3AR^{-/-}$ and $IL\text{-}10^{-/-}$ mice were used: animals underwent CCI of sciatic nerve and then were reconstituted with an intravenous injection of T cells isolated from wild type or different transgenic donors. Onset and development of mechano-allodynia was followed up using Von Frey filaments before and after A_3AR agonists systemic and intrathecal administration.

II) Investigation of the modulation of prokineticin system following CCI in PKR1 and PKR2 knock out mice

It has been recently reported that prokineticin system is strongly involved in the onset and progression of neuropathic pain. In two different animal models (CCI and SNI) treatment with selective antagonists of prokineticins' receptors (PKRs) block and reverse neuropathic pain (Lattanzi et al., 2015; Maftei et al., 2014). It has been demonstrated that Prokineticin 2 (PROK2), up-regulated in

injured sciatic nerve, promotes the recruitment and survival of infiltrating granulocytes and macrophages inducing their shift to a pro-inflammatory phenotype by increasing the production of IL-1β (Franchi et al., 2008; Martucci et al., 2006) and blocking the release of anti-inflammatory and neuroprotective cytokine IL-10 (Lattanzi et al., 2015; Maftei et al., 2014). Moreover, PROK2 contributes to peripheral sensitization also by directly activating PKRs expressed on sensitive neurons: this leads to a reduction of nociceptive threshold thanks to the engaging of TRPV1 and TRPA1 signalling. Treatment with selective antagonists of PKRs restores physiological levels of IL-10 in both sciatic nerve and DRGs (Maftei et al., 2014) and prevents the hyperactivation of astrocytes and microglia at spinal cord level, promoting the anti-inflammatory phenotype (Guida et al., 2015). All these evidences indicate PKRs as good pharmacological targets for chronic pain management. However, several aspects of the modulation and the expression of PKRs in pathological conditions remain still unclear.

Previous studies from our laboratory have demonstrated how mice lacking of prokineticin receptors show an impaired nociception and inflammatory pain sensation when compared with WT littermates (Negri et al., 2006). Knock out animals are an incredibly useful tool in pharmacological research, so, based on this literature, we decided to investigate the behavioural responses induced by peripheral nerve ligation in both $PKR1^{-/-}$ and $PKR2^{-/-}$ mice, to better characterize the contribution of each receptor in neuropathic pain development.

For this study, wild type $PKR1^{-/-}$ and $PKR2^{-/-}$ male mice underwent CCI surgery. Onset and development of mechano-allodynia and thermal hyperalgesia were followed up using Von Frey filaments and Plantar test, respectively. Lumbar region of spinal cord was harvested for immunofluorescence assay.

3. RESULTS AND DISCUSSION

3.1 A_3 adenosine receptor (A_3AR) agonists reverse neuropathic pain through a $CD4^+$ T cell-dependent IL-10 pathway

3.1.1 Antiallodynic effects of A_3AR agonists are completely lost in both $Rag1^{-/-}$ and IL-$10^{-/-}$ mice

Our first hypothesis was that T cells and IL-10 could be important contributors in the mechanism of action of A_3AR agonists. To assess this, we used mice lacking of mature T and B cells ($Rag1^{-/-}$) or IL-10 deficient mice (IL-$10^{-/-}$). After CCI surgery, C57BL/6 wild type (WT), $Rag1^{-/-}$ and IL-$10^{-/-}$ mice displayed identical onset and maximal severity of mechano-allodynia in ipsilateral paw. In accordance with previous findings (Chen et al., 2012), acute systemic administration of A_3AR agonists during peak CCI-induced neuropathic pain (day 7-day 8 after surgery) completely reversed mechano-allodynia in WT mice, with a fast onset of action (<30 min) and full efficacy within 1 h post-dosing, as shown in Fig 1A. However, A_3AR agonists completely lost their ability to reverse CCI-induced mechano-allodynia when administered i.p. both in $Rag1^{-/-}$ and IL-$10^{-/-}$ mice (Figs. 16A and 16C). In order to provide a control experiment, on Day 9 after surgery WT and $Rag1^{-/-}$ mice were treated with morphine (3mg/kg s.c.). Anti-allodynic effects of morphine were not affected in $Rag1^{-/-}$ mice (Figs. 16E and 16F), showing that $Rag1^{-/-}$ mice are able to respond to analgesics whose mechanisms are known not to act via T or B lymphocytes. These findings gave us a first important indication: the efficacy of A_3AR agonists in reversing neuropathic pain is T cell-mediated and involves the release of endogenous IL-10.

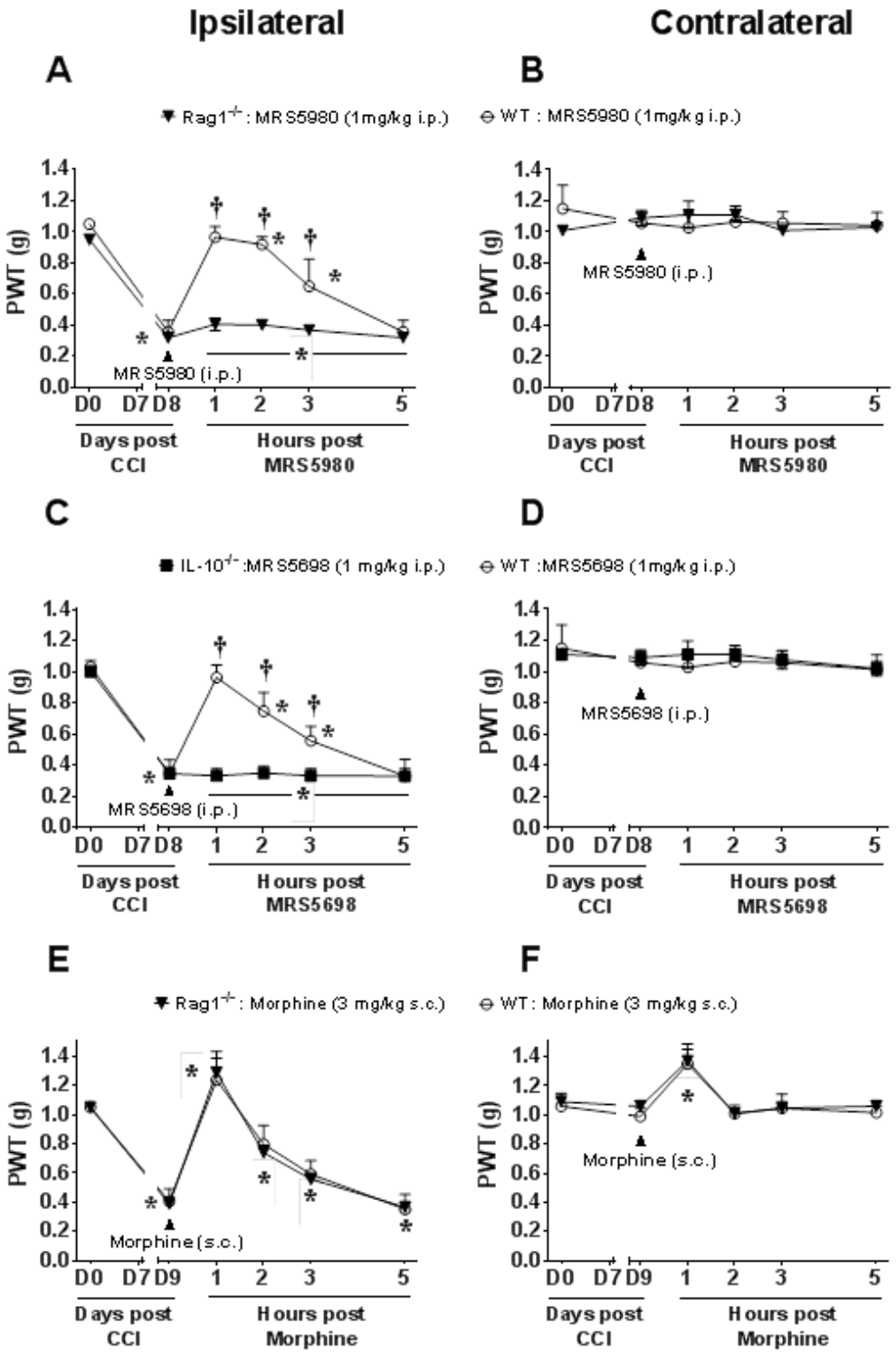

Fig 16. A_3AR agonists lost their ability to reverse CCI-induced neuropathic pain in both Rag1$^{-/-}$ or IL-10$^{-/-}$ mice. CCI-induced mechano-allodynia (manual Von Frey filaments) through Day 8 in Rag1$^{-/-}$ (▼), IL-10$^{-/-}$ (■) and C57BL/6 wild type (⊖) mice. Administration of MRS5980 (1 mg/kg; i.p.) or MRS5698 (1 mg/kg; i.p.) on Day 8 after surgery reversed mechano-allodynia in ipsilateral paws (**A**, **C**) of wild type, but not in Rag1$^{-/-}$ or IL-10$^{-/-}$ mice. A_3AR agonists' administration did not affect contralateral PWT (g) (**B**, **D**). Anti-allodynic effects of morphine (3mg/kg, s.c.) were not affected in Rag1$^{-/-}$ mice (**E**, **F**). Data are mean ± SD for n=5-7 mice/group; *p < 0.05 vs D0 by two-way ANO VA with Dunnett's test; †p < 0.001 vs Rag1 $^{-/-}$ or IL10 $^{-/-}$ by two-way ANOVA with Bonferroni.

3.1.2 A_3AR agonists' beneficial effects are mediated by T lymphocytes activation

To provide evidence that A_3AR agonists' beneficial effects are effectively T-cells mediated, we adoptively transferred $CD3^+$ T cells, isolated from spleens and lymph nodes of C57BL/6 WT mice, to $Rag1^{-/-}$ mice which underwent CCI surgery. T cells or vehicle were injected intravenously into the tail veins of $Rag1^{-/-}$ and WT mice one day before MRS5980 (1 mg/kg i.p.) treatment. Baseline values measured on day 8 showed how $CD3^+$ T cell transfer alone did not influence mice pain threshold after CCI surgery (Fig 17). Following MRS5980 administration, as expected, $Rag1^{-/-}$ mice that did not receive $CD3^+$ T cells were not able to reverse CCI-induced mechano-allodynia (Fig 17A); on the contrary, $Rag1^{-/-}$ mice reconstituted with $CD3^+$ T cells gained the ability to respond to MRS5980 at rates comparable to WT mice (Fig 17B). With these results we confirmed that A_3AR agonists' beneficial effects rely on the modulation of inflammatory process through the involvement of T cells and the enhancement of their immune response.

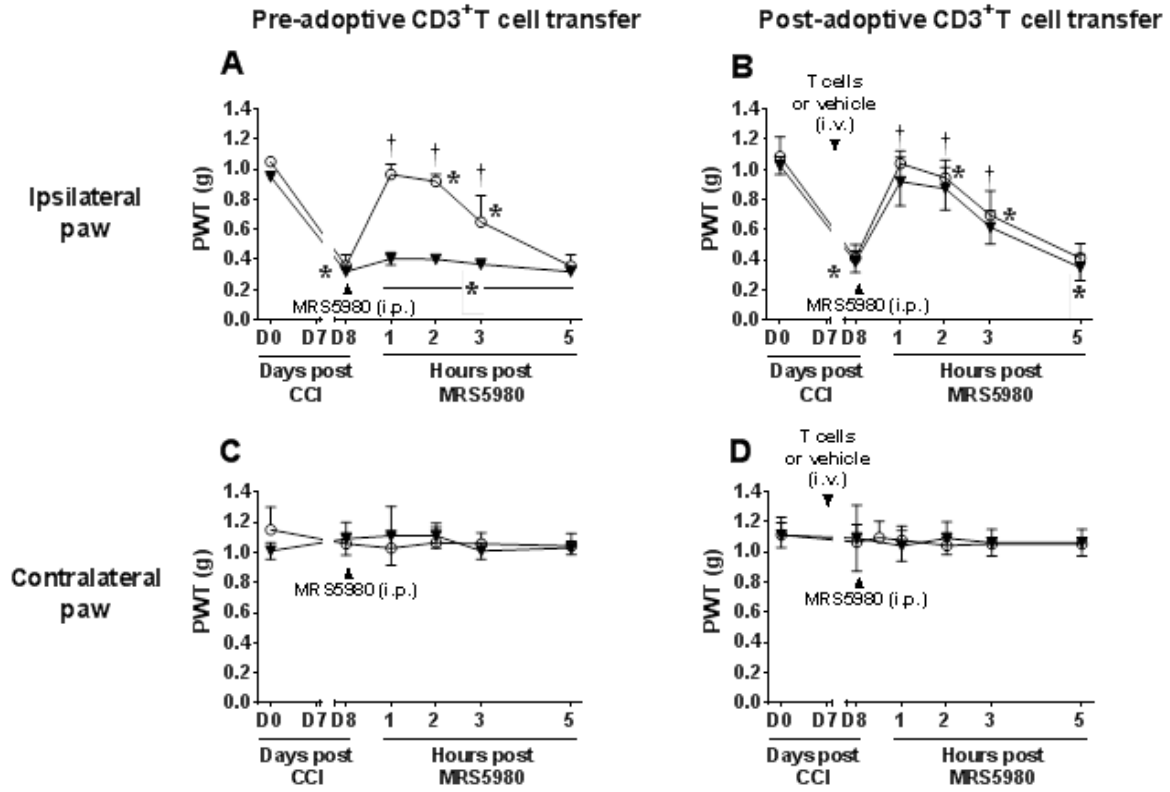

Fig 17. Adoptive $CD3^+$ T cells transfer restored A_3AR agonists' beneficial effects in $Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice (▼) are not able to respond to A_3AR agonists after CCI surgery (**A**). But, following $CD3^+$ T cell transfer, they gained the ability to respond to MRS5980 and reversed mechano-allodynia in ipsilateral paws at rates comparable to WT mice (⊖) (**B**). MRS5980 treatment or $CD3^+$ T cell transfer did not affect contralateral PWT (g) (**C**, **D**). Data are mean ± SD for n=5-7 mice/group; *p < 0.05 vs D0, †p < 0.001 vs D8 by two-way ANOVA with Dunnett's test.

To further explore the key players responsible for A_3AR agonists' beneficial effects, we then focused our research on the identification of the T cells' subset involved in our drugs' mechanisms of action. WT and $Rag1^{-/-}$ mice received either $CD4^+$ T cells, $CD8^+$ T cells or vehicle intravenously on day 7 after CCI surgery. Acute intraperitoneal administration of MRS5980 on day 8 reversed, also if not completely, mechano-allodynia in $Rag1^{-/-}$ mice receiving $CD4^+$ T cells (Fig 18A). In contrast, mechano-allodynia persisted in mice that were transferred with only $CD8^+$ T cells (Fig 18C). These data showed how $CD4^+$ T cells are the T cell subset mainly involved in A_3AR agonists' action.

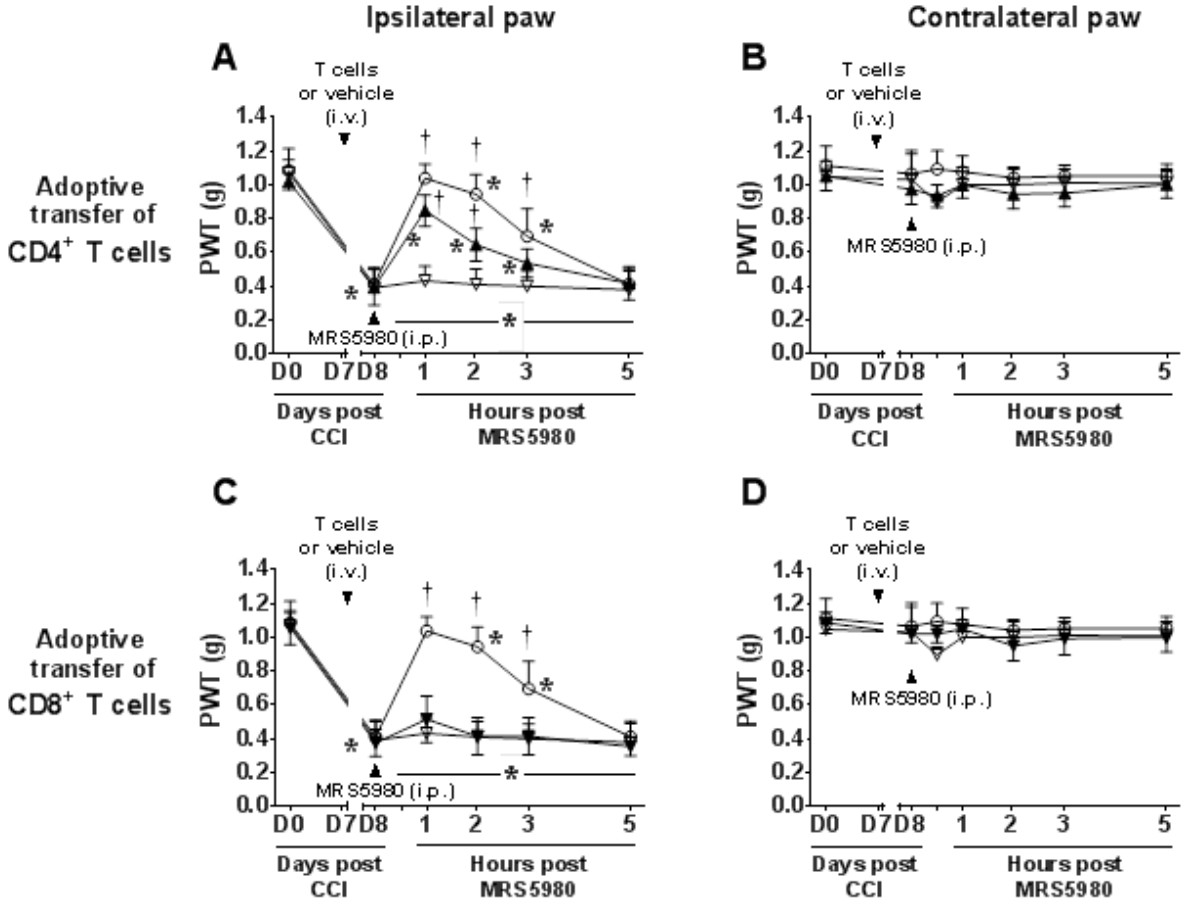

Fig 18. Identification of T cells subset mainly responsible for A_3AR agonists' beneficial effects. (**A**, **C**) $CD4^+$ (▲), $CD8^+$ (▼) T cells or vehicle (▽), were injected i.v. to $Rag1^{-/-}$ mice one day before MRS5980 i.p. administration. $Rag1^{-/-}$ mice receiving $CD4^+$ T cells reversed mechano-allodynia but not at rates comparable to WT mice (⊖), whereas $Rag1^{-/-}$ mice reconstituted with $CD8^+$ T cells were not able to respond to MRS5980. MRS5980 treatment or T cell transfer did not affect contralateral PWT (g) (**B**, **D**). Data are mean ± SD for n=5-7 mice/group; *p < 0.05 vs D0 by two-way ANOVA with Dunnett's test; †p < 0.001 vs $Rag1^{-/-}$ (vehicle) or $Rag1^{-/-}$ (vehicle/$CD4^+$/$CD8^+$) or WT by two-way ANOVA with Turkey comparisons.

To investigate if in CCI model A_3AR agonists can directly modulate T cells' activity, we used two different approaches. In the first one, $A_3AR^{-/-}$ mice were used as donors of $CD4^+$ T cells. On day 7 after surgery, $Rag1^{-/-}$ mice received an i.v. injection of vehicle or $CD4^+$ T cells previously isolated from $A3AR^{-/-}$ mice, so in this case in recipient mice the receptor is express throughout the body except on $CD4^+$ T cells. Acute intraperitoneal administration of MRS5980 on day 8 had no effect on ipsilateral paw withdrawal threshold (PWT) of $Rag1^{-/-}$ mice compared to the complete reversal of mechano-allodynia observed in WT mice (Fig 19A). This first result clearly proves a direct action of the A_3AR agonists on A_3AR expressed on $CD4^+$ T cells.

Through the second approach, we wanted to examine the specificity of MRS5980 action. In this experiment, $A_3AR^{-/-}$ mice underwent CCI surgery and 7 days later they received an i.v. injection of $CD4^+$ T cells isolated from WT donors. In this case in recipient mice A_3AR is expressed only on the membrane of $CD4^+$ T cells. Acute intraperitoneal administration of MRS5980 on day 8 totally reversed established mechano-allodynia in $A3AR^{-/-}$ mice receiving $CD4^+$ T cells as well as in WT mice (Fig 19C). As expected, no effect was observed in the control group. Contralateral PWT did not differ from baseline at any time point (Fig 19 B, D).

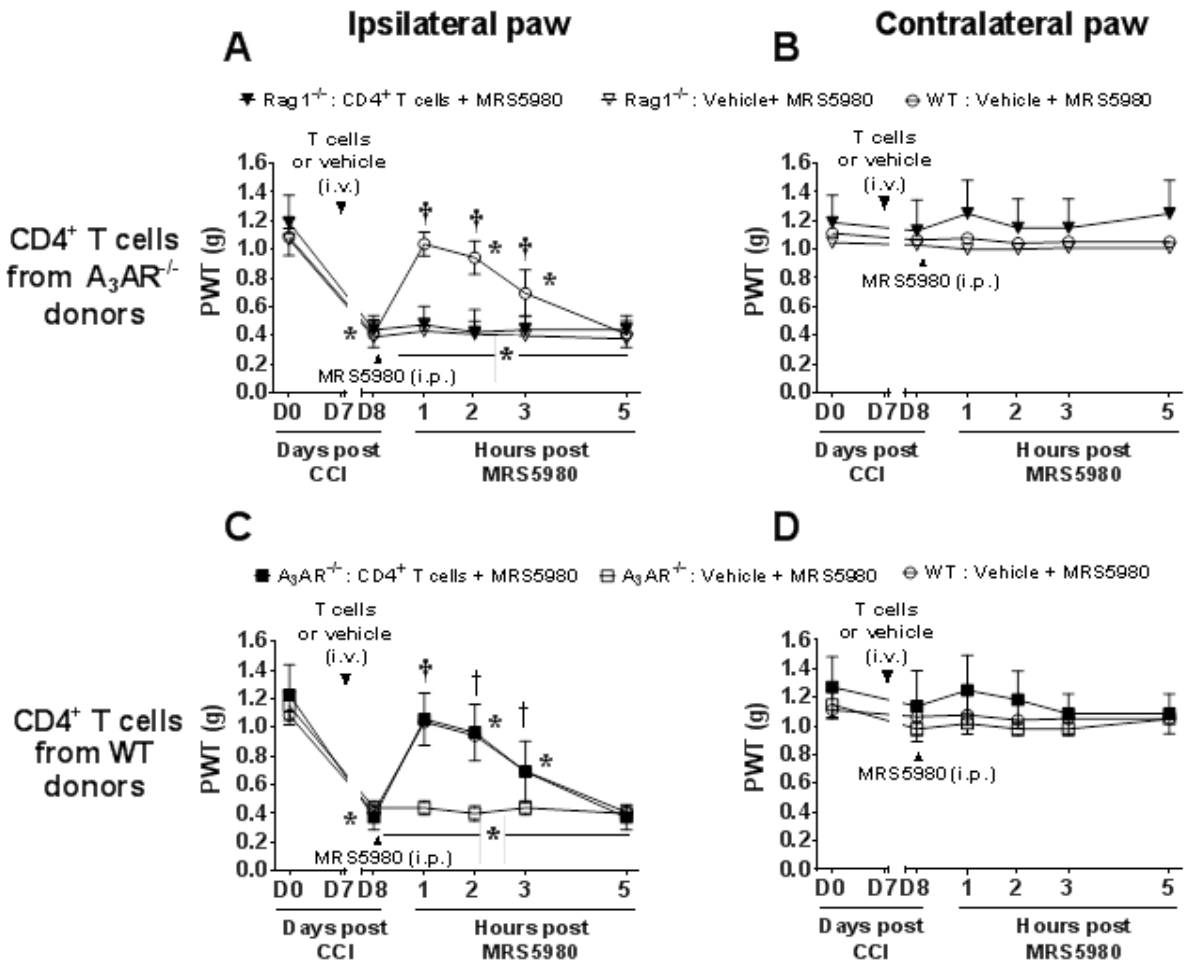

Fig 19. The action of the A_3AR agonist MRS5980 is mediated by A_3AR expressed on $CD4^+$ T cells. $CD4^+$ T cells isolated from $A_3AR^{-/-}$ donors (▼) or vehicle (▽), were injected i.v. to $Rag1^{-/-}$ mice one day before MRS5980 i.p. administration. $Rag1^{-/-}$ mice reconstituted with $CD4^+$ T cells were not able to respond to MRS5980 (**A**). $A_3AR^{-/-}$ mice received either wild type $CD4^+$ T cells (■) or vehicle (□) on day 7 after CCI. The following day, $A_3AR^{-/-}$ mice reconstituted with $CD4^+$ T cells completely reversed CCI-induced mechano-allodynia after MRS5980 i.p. administration (**C**). MRS5980 treatment or T cell transfer did not affect contralateral PWT (g) (**D**, **B**). Data are mean ± SD for n=5-7 mice/group; *$p < 0.05$ vs D0 and †$p < 0.001$ vs D8 by two-way ANOVA with Dunnett's test.

3.1.3 Investigation of $CD4^+$ T cells site of action

To investigate the site of action of A_3AR agonists, on day 9 after surgery MRS5980 (3nmol) was administered intrathecally (i.t) to both WT and $Rag1^{-/-}$ mice reconstituted with T cells. Similarly to results obtained with intraperitoneal administration, $Rag1^{-/-}$ mice reconstituted with $CD3^+$ T cells rapidly reversed mechano-allodynia at rates comparable to WT mice. Beneficial effects of MRS5980 were evident, though slightly attenuated, in $Rag1^{-/-}$ mice reconstituted only with $CD4^+$ T cells subset, whereas the degree of reversal observed in mice that received $CD8^+$ T cells was minimal (Fig 20).

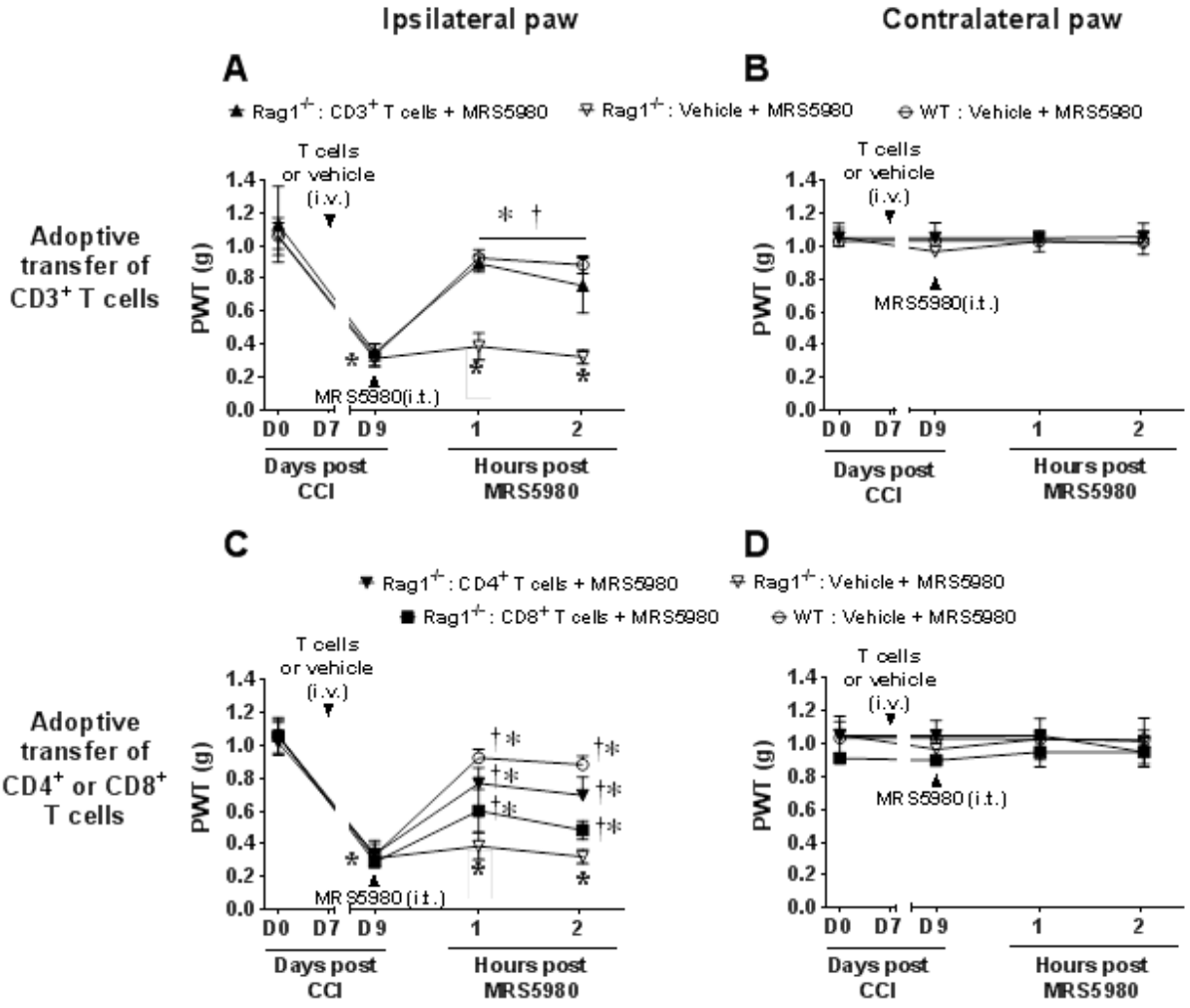

Fig 20. Investigation of a possible A_3AR agonists' site of action. $CD3^+$ (▲), $CD4^+$ (▼), $CD8^+$ (■) T cells or vehicle (▽), were injected i.v. to $Rag1^{-/-}$ mice two days before MRS5980 (3nmol; i.t.) administration. $Rag1^{-/-}$ mice receiving $CD3^+$ T cells reversed CCI-induced mechano-allodynia at rates comparable to WT mice (⊖) (**A**), whereas in mice reconstituted with $CD4^+$ or $CD8^+$ T cells MRS5980 beneficial effects were evident, but not complete, or minimal respectively (**B**). MRS5980 treatment or T cell transfer did not affect contralateral PWT (g) (**B**, **D**). Data are mean ± SD for n=5-7 mice/group; *$p < 0.05$ vs D0; †$p < 0.001$ vs D8 by two-way ANOVA with Dunnett's test.

These results obtained in Rag1$^{-/-}$ mice suggest that A_3AR agonists act on activated T cells which infiltrate DRGs and/or spinal cord in response to molecular signals to modulate inflammation. To verify this hypothesis, we stained CD4$^+$ T cells in the lumbar sections of spinal cord and in DRGs. Immunofluorescence analysis showed CD4$^+$ T cells infiltration in the DRGs of Rag1$^{-/-}$ mice reconstituted with CD4$^+$ T cells from WT donors, compared to Rag1$^{-/-}$ received the vehicle (data in progress). In particular, our data demonstrated that following CCI, A_3AR agonists' administration evokes trafficking of immune cells from circulation into the DRGs and that these neuroimmune interactions play a critical role in the therapeutic effects of A_3AR agonists in alleviating neuropathic pain.

3.1.4 CD4$^+$ T cells are the source of IL-10 needed for A_3AR agonists' beneficial effects

Our findings demonstrated that CD4$^+$ T cells are key players in A_3AR agonists' mechanism of action. CD4$^+$ T cells are known to be essential for the resolution of inflammatory process through the release of IL-10. Since our very first results (Fig 16C) showed that A_3AR agonists' beneficial effects are strictly dependent on endogenous release of this interleukin, we decided to investigate the cellular source of IL-10. On day 7 after CCI surgery, Rag1$^{-/-}$ mice received i.v. vehicle or CD4$^+$ T cells, either from WT or IL-10$^{-/-}$ mice donors. As we already shown, adoptive transfer of CD4$^+$ T cells from WT donors completely restored the beneficial effects of A3AR agonists in Rag1$^{-/-}$ mice (Fig 21A). However, CD4$^+$ T cells from IL-10$^{-/-}$ mice donors provided no restoration (Fig 21C). This result proved that A_3AR agonists reverse CCI-induced mechano-allodynia through a CD4$^+$ T cells-dependent IL-10 pathway.

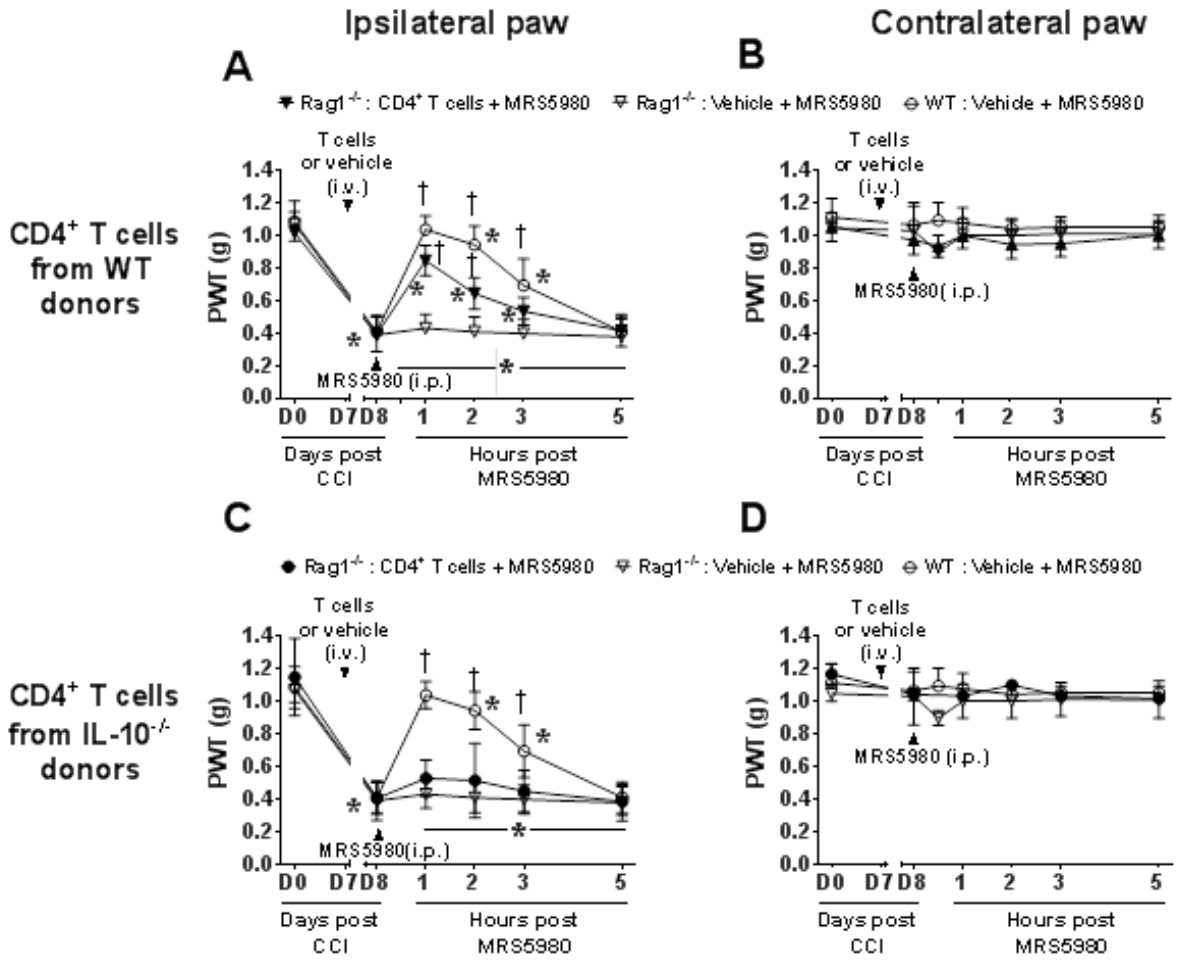

Fig 21. A_3AR agonists reverse CCI-induced mechano-allodynia through a $CD4^+$ T cells-dependent IL-10 pathway. $Rag1^{-/-}$ mice were transferred with $CD4^+$ T cells isolated from either WT (▼) (**A**), or IL10-/- (●) (**C**) donors. $Rag1^{-/-}$ mice transferred with $CD4^+$ T cells from WT donors reversed mechano-allodynia also if not at rates comparable to WT mice (⊖). On the contrary, $Rag1^{-/-}$ mice transferred with $CD4^+$ T cells from $IL10^{-/-}$ donors completely lose the ability to respond to A_3AR agonist as well as $Rag1^{-/-}$ mice injected i.v. with vehicle (▽). Data are mean ± SD for n=5-7 mice/group; *p < 0.05 vs D0; †p < 0.001 vs D8 by two-way ANOVA with Dunnett's test.

3.1.5 Discussion

In this study, using a well-characterized model of chronic neuropathic pain resulting from constriction of the sciatic nerve in mice (Bennett and Xie, 1988), we have demonstrated for the first time that A_3AR agonists are able to reverse established mechano-allodynia through a T cell driven pathway. A_3AR is expressed on the membrane of different immune cells (Borea et al., 2015) and its expression resulted increased in pathological conditions, in correlation with the progression of inflammatory response (Ochaion et al., 2009), suggesting that its activation could be directly involved in immune cells' activities. Our first results reported how A_3AR agonists' beneficial effects are completely lost in mice deficient of mature B and T cells ($Rag1^{-/-}$). However, following $CD3^+$ T cells adoptive transfer, $Rag1^{-/-}$ animals gain the ability to respond to A_3AR agonists and completely reverse neuropathic pain at rates comparable with wild type mice. We further investigated the different T cells' subtypes involved in this mechanism and we identified the $CD4^+$ T cells as the major contributor in the analgesic effects induced by A_3AR agonists administration. The balance of specific cytokines secreted by $CD4^+$ and $CD8^+$ T cells determines the outcome of the inflammatory response after injury (Neurath, 2014). Targeting immune cells to modulate cytokines profile is a winning strategy to control the progress of inflammatory process thus preventing its chronicization, which represent the main trigger for the establishment of neuropathic pain states. It has been reported that $CD4^+$ T cells activity is critical for alleviating neuropathic pain (Austin et al., 2012; Ji et al., 2016). Here we confirmed the crucial role of these cells in modulating anti-inflammatory response, demonstrating that this T cell subset is the one mainly involved in A_3AR agonists' mechanism of action. Interestingly, it has been recently reported that also activated cytotoxic $CD8^+$ T cells have a protective role in different models of chronic inflammatory pain (Baddack-Werncke et al., 2017; Krukowski et al., 2016). The switch of $CD8^+$ T cells to a cytotoxic phenotype is largely due to $CD4^+$ T cells through the release of Interleukin-2 (IL-2) and the involvement of dendritic cells (Boyman and Sprent, 2012; Mailliard et al., 2002; Melief, 2013). Therefore, it is possible that $CD8^+$ T cell adoptive transfer in $Rag1^{-/-}$ mice was not sufficient to elicit A_3AR agonists' effects because they failed their complete activation in absence of $CD4^+$ T cell. However, also if $CD8^+$ T cells could partially contribute to the final effects of our drugs, our data clearly showed that $CD4^+$ T cells are the driving force in A_3AR agonists' action.

MRS5980, the A_3AR agonist used in this study, was recently developed and characterized (Fang et al., 2015; Tosh et al., 2015a). We have shown how anti-nociceptive effects of this drug are lost in $A_3AR^{-/-}$ mice, confirming its high specificity at A_3AR. More interestingly, however, was the demonstration that, in this model, its beneficial effects in abrogate neuropathic pain are exclusively due to its action on a specific subpopulation of T lymphocytes. When systemically administered to neuropathic $A_3AR^{-/-}$ mice previously transferred with wild type $CD4^+$ T cells, in fact, the anti-allodynic effects of MRS5980 were comparable to the results obtained in wild type mice. Together with the data obtained in $Rag1^{-/-}$ mice, these results demonstrate that the presence of A3 receptor on $CD4^+$ T cells is necessary and sufficient to elicit a complete analgesic response to the drug.

To corroborate this assumption, we have also reported that in $Rag1^{-/-}$ mice intrathecal injection of A_3AR agonists did not evoke an analgesic response, demonstrating that cells in DRGs or in the spinal cord, such as astrocytes, are not a predominant target for our drug in this model.

A_3AR activation is associated to protective effects throughout the organism, since it enhances physiological functions in almost all tissues (Borea et al., 2015). However, prolonged stimulation or the usage of high drug doses for a long-term treatment, could lead to the appearance of undesired side effects. For this reason, the possibility to obtain a great analgesic effect by intrathecal administration, as the one observed with our agonist, can decrease the side effects typically associated with oral or parental drug delivery and may allow for better quality of life outcomes in patients with chronic pain.

In this work we have also shown how, following peripheral nerve injury, $CD4^+$ T cells infiltrate the DRGs where are the specific target of A_3AR agonists. Many studies have been performed, using different neuropathic pain models, to investigate the contribute of lymphocyte infiltration to the development and the maintenance of persistent pain, but this phenomenon remains somewhat controversial (Gattlen et al., 2016; Walsh et al., 2015). T cell infiltration into spinal cord and DRGs has been reported as contributor to mechanical hypersensitivity in several pain models including CCI and spinal nerve ligation/transection (Costigan et al., 2009; Grace et al., 2011; Hu and McLachlan, 2002). These infiltrating lymphocytes were found to be constituted predominantly by $CD4^+$ T cells (Cao and DeLeo, 2008) which are known to be the major player in the adaptive immune response following injury. In our experiments we have shown that $Rag1^{-/-}$ mice develop the same level of hypersensitivity of WT mice after CCI and we found no evidence of the fact that $Rag1^{-/-}$ mice could experience a stronger pain sensation following T cells' injection. So, in our hands, there are no evidence for a role of T cells in the modulation of nociception. Thorough analysis, both *in vitro* and *in vivo*, have demonstrated that the dominant $CD4^+$ T cell subtype involved in this mechanism are the

T helper 1 (Th1) cells, which contributes to the establishment of chronic inflammation in a microglia-independent manner, via a mechanism that could involve multiple Th1 inflammatory cytokines release and astrocytic activation (Draleau et al., 2014; Moalem et al., 2004). On the contrary, our findings, clearly evidence that A_3AR agonists act on a subpopulation of $CD4^+$ T cell able to produce and release IL-10, such as T helper 2 (Th2) or T regulatory (Tregs) cells. The strategy of enhance infiltrating T cells activity to alleviate persistent pain has been also reported by Leger and colleagues: Glatiramer, a synthetic amino acid polymer that is known to induce the development of Th2 and enhance the production of IL-10 (Aharoni et al., 2003), when administered systemically reversed neuropathic allodynia and increased IL-10 expressing T cells in neuropathic dorsal horns (Leger et al., 2011).

Further, we have proven that A_3AR agonists' beneficial effects strictly depend on the release of IL-10, since they are completely lost in IL-$10^{-/-}$ animals, confirming the IL-10 downstream of A_3AR activation previously reported (Wahlman et al., 2018). In particular, our results demonstrate that A_3AR agonists are able to reverse CCI-induced neuropathic pain by modifying cytokines' profile released during inflammation process, acting directly on CD4+ T cells and inducing the release of IL-10. It has been widely reported that IL-10 suppresses cellular immunity and inhibits the synthesis and release of pro-nociceptive mediators such as TNFα, IL1-β, IL-6, IL-8 and IL-12 (Howard and O'Garra, 1992; Ledeboer et al., 2002; Moore et al., 2001). More recently, in addition to its anti-inflammatory effects, it has been demonstrated that IL-10 exerts also direct effects on neurons. Expression of IL-10 receptor mRNA and protein has been found in spinal cord neurons *in vitro* where IL-10 activates signalling pathways involved in neuroprotection and growth to overcome the neurotoxic effects of glutamate (Zhou et al., 2009b). In vivo studies, in a model of spinal cord injury, confirmed that IL-10 promotes neuronal survival and improves motor function (Zhou et al., 2009a). In neurons, the effects of IL-10 against glutamate-induced excitotoxicity are mediated through Jak-Stat3 and PI3K/AKT pathways, both involved in the transcription of survival genes and intracellular Ca^{2+} levels normalization (Sharma et al., 2011; Turovskaya et al., 2012; Zhou et al., 2009b). In DRG it has been demonstrated that IL-10, acting through IL-10R1 receptor, down-regulates the over-expression of voltage-gated sodium channels reducing the hyperexcitability of DRG neurons induced by nerve injury (Shen et al., 2013). This data could explain the rapid analgesic effects that we observed following A_3AR agonists administration and, interestingly, they also correlate with our previous findings which reported how A_3AR activation in CCI animals inhibits nociceptive processing by reducing spinal neuronal excitability (Little et al., 2015).

In summary, we have demonstrated for the first time that treatment with A_3AR agonists following peripheral nerve injury has surprising therapeutic results that converge in considerably attenuating abnormal nociception. This study suggests A_3AR agonists therapy, which has already been proven safe in the clinic in other inflammatory diseases (David et al., 2016; Stemmer et al., 2013), may provide a novel treatment also for chronic neuropathic pain.

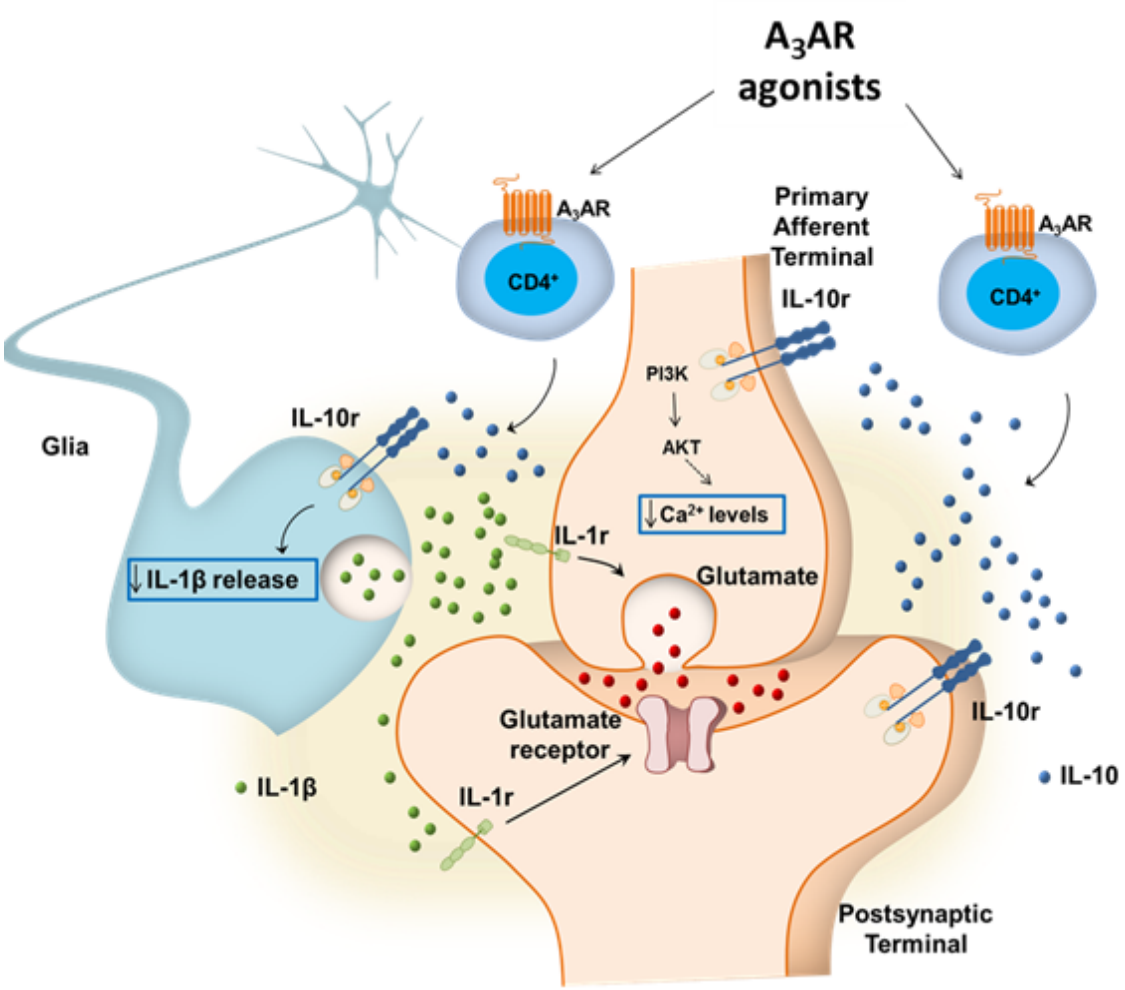

Fig 22. Schematic representation of a possible mechanisms underlying A_3AR-induced anti-nociception. A_3AR agonists, acting specifically on the receptor expressed by $CD4^+$ T cells, induce the release of IL-10. The activation of IL-10 receptors on glial cells inhibits the release of pro-inflammatory cytokines such as IL-1β, whereas the activation of neuronal IL-10 receptors, restores intracellular calcium levels, reducing neuronal excitability (Ledeboer et al., 2002; Sharma et al., 2011; Turovskaya et al., 2012).

3.2. New insight in the role of prokineticin system in neuropathic pain: TM4-7 involvement the development of contralateral neuropathy in PKR1$^{-/-}$ mice

3.2.1. CCI-induced neuropathic pain in WT, PKR1$^{-/-}$ and PKR2$^{-/-}$ mice: the discovery of contralateral neuropathy

Previous work from our group, have demonstrated a central role of the prokineticin system in the development and maintenance of neuropathic pain. Sciatic nerve ligation induces an overexpression of PROK2 which is involved in peripheral inflammation through the recruitment of neutrophils and macrophages, and in central sensitization since it contributes to spinal glia activation and aberrant excitation of projection neurons in the dorsal horn (Lattanzi et al., 2015; Maftei et al., 2014). PKRs expression has been also investigated in neuropathic WT mice: nerve damage induces a strong overexpression of PKR2 both in peripheral and central nervous system, whereas PKR1 significantly increase only in injured sciatic nerve (Negri and Maftei, 2018).

Based on these previous observations, we decided to investigate the behavioural responses induced by peripheral nerve ligation in both PKR1$^{-/-}$ and PKR2$^{-/-}$ mice, to better characterize the contribution of each receptor in neuropathic pain development. Following CCI surgery, thermal hyperalgesia (plantar test) and mechano-allodynia (Von Frey filaments) were followed up for more than 3 months. WT and PKR2$^{-/-}$ mice displayed identical onset and maximal severity of thermal hyperalgesia and mechano-allodynia in ipsilateral paws. PKR1$^{-/-}$ mice, instead, developed a strong hypersensitivity only on contralateral paws, showing hyperalgesia and allodynia at rates comparable to the ones observed in ipsilateral paws of the other two experimental groups (Fig 23).

Thermal hyperalgesia was already evident in ipsilateral paws of WT and PKR2$^{-/-}$ and in contralateral paws of PKR1$^{-/-}$ mice 3 days after CCI (Fig 23A). By post-CCI day 45-50 all animals showed a gradual increase in thermal nociceptive threshold, until the complete recovery 55 days after surgery. Interestingly, in PKR2$^{-/-}$ mice recovery was slower and thermal hyperalgesia still persisted, also if attenuated, until 70-80 days after CCI. Starting from post-CCI day 10, mechanical nociceptive threshold (Fig 23B) decreases of 40%–50% in the ipsilateral compared to contralateral hind paws in both WT and PKR2$^{-/-}$ mice, whereas PKR1$^{-/-}$ mice developed allodynia on contralateral hind paws. By post-CCI day 45-50, in both WT and PKR2$^{-/-}$ mice allodynia gradually decreased and there was a

significant increase of the mechanical threshold, until the complete recovery 70 days after surgery. In PKR1$^{-/-}$ mice mechano-allodynia still persisted, also if slightly attenuated, until 100 days after CCI.

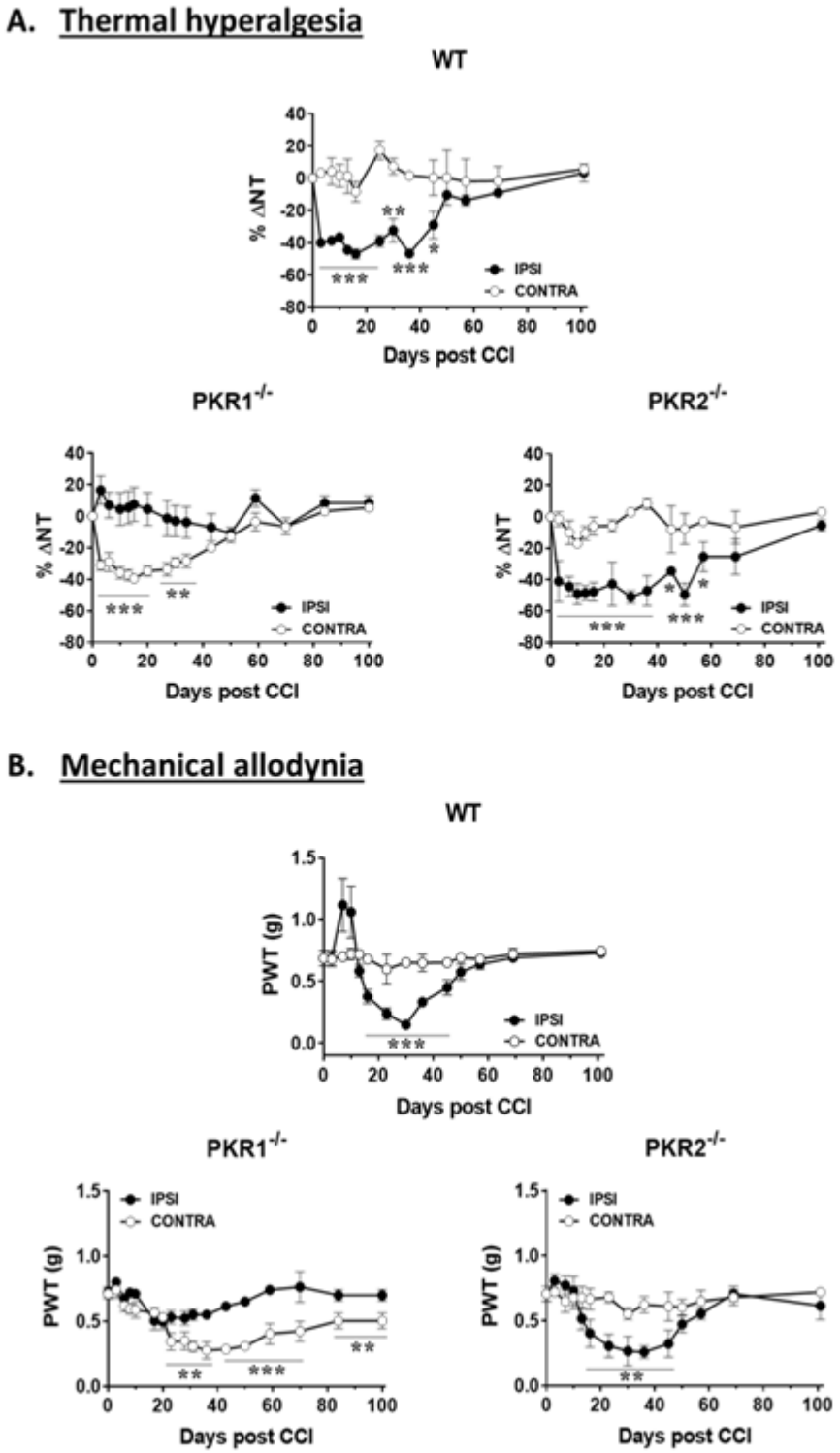

Fig 23. PKR1$^{-/-}$ mice developed contralateral neuropathy following CCI surgery. Time-course of CCI-induced thermal hyperalgesia (**A**, plantar test) and mechano-allodynia (**B**, manual Von Frey filaments) in WT, PKR1$^{-/-}$and PKR2$^{-/-}$ mice. Values are referred to measurements on hind paws ipsilateral (●) and contralateral (⊖) to the injury. Data are mean ± SD for n=5-7 mice/group; *p < 0.05, **p < 0.01 and ***p < 0.001 vs contralateral paws by two-way ANOVA with Bonferroni.

3.2.2. Investigation of prokineticin system alterations following CCI-induced neuropathic pain

Considering the results obtained by behavioural tests, we decided to investigate the expression of PROK2 and PKR2 in PKR1$^{-/-}$ mice in comparison with WT animals.

As expected, immunofluorescence analysis (Fig 24A) performed on mice sacrificed 10 days after CCI, showed a strong overexpression of PROK2 in both WT and PKR1$^{-/-}$ mice in ipsilateral dorsal horn compared to its faint expression in sham animals.

An interesting data, instead, came out by observing the contralateral dorsal horns. A very low expression of PROK2 was detected in WT mice, confirming previous findings (Maftei et al., 2014) but, curiously, high levels of PROK2 were present in dorsal horns of PKR1$^{-/-}$ mice (Fig 24B).

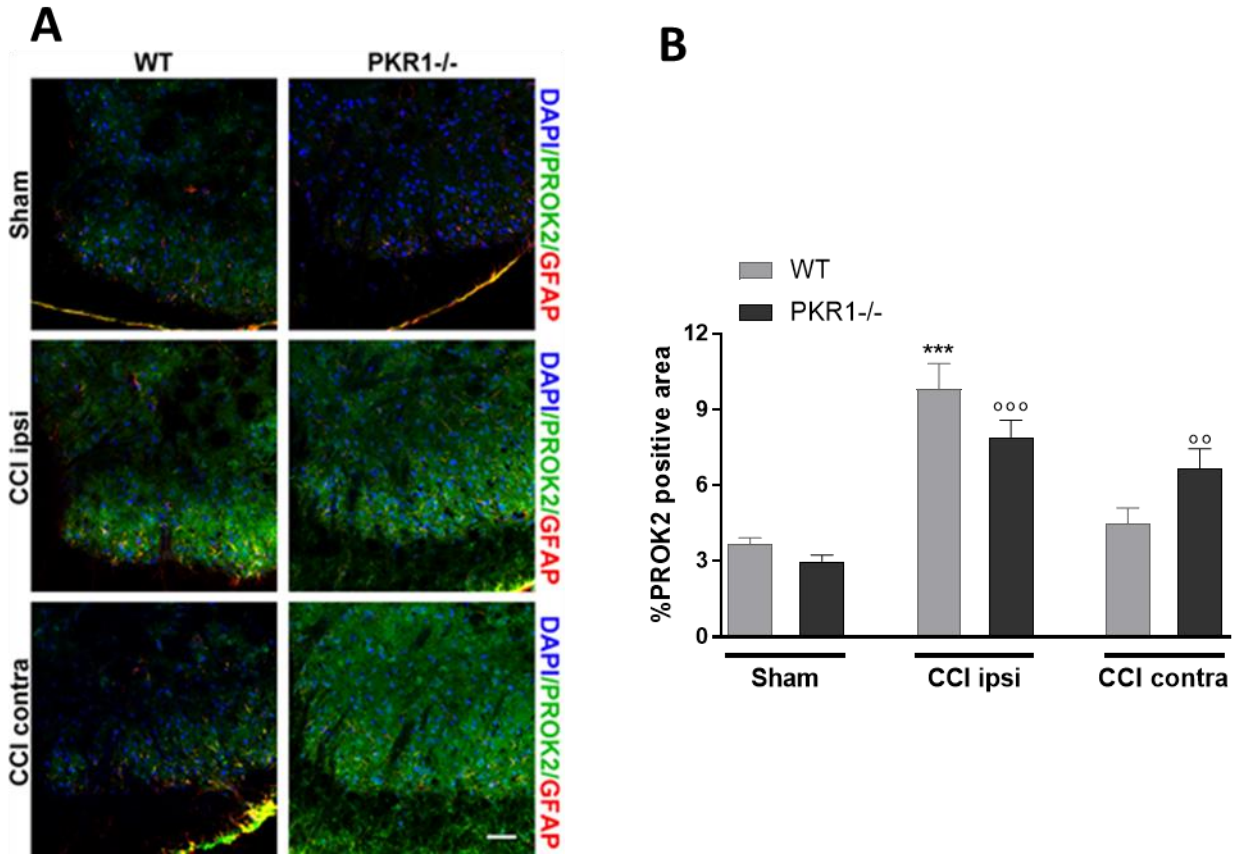

Fig 24. PKR1$^{-/-}$ mice, following CCI surgery, shows high levels of PROK2 in both ipsilateral and contralateral dorsal horn. A) PROK2 positive profiles (green) and GFAP (astrocytes marker) positive profile (red) in WT and PKR1$^{-/-}$ mice. **B)** Quantification of PROK2 in the spinal cord. Sciatic nerve ligation induced a substantial increase of PROK2 signal 10 days after surgery in ipsilateral dorsal horn of both WT and PKR1$^{-/-}$ mice. However, elevate expression of PROK2 was also detected in contralateral dorsal horn of PKR1$^{-/-}$ mice, whereas, as expected, almost no signal was detected in WT mice. Cell nuclei were counterstained with DAPI (blue). Scale bar: 50µm.
***$p < 0.001$ vs WT sham; °°° $p < 0.001$ and °°$p < 0.01$ vs KO sham by one-way ANOVA with Tukey's.

PKR2 expression has been also investigated. As showed in figure 25, immunofluorescence staining revealed a faint PKR2 immunoreactivity in both WT and PKR1$^{-/-}$ sham mice. 10 days after CCI, PKR2 immunofluorescence was strongly increased in the ipsilateral dorsal horn of both WT and PKR1$^{-/-}$ mice, mainly associated with neuronal cells (NeuN-positive cells, in red). No differences were observed in the expression of PKR2 between WT and PKR1$^{-/-}$ neuropathic mice in contralateral dorsal horns: in both experimental groups the receptor result not modulated in this area after the surgery.

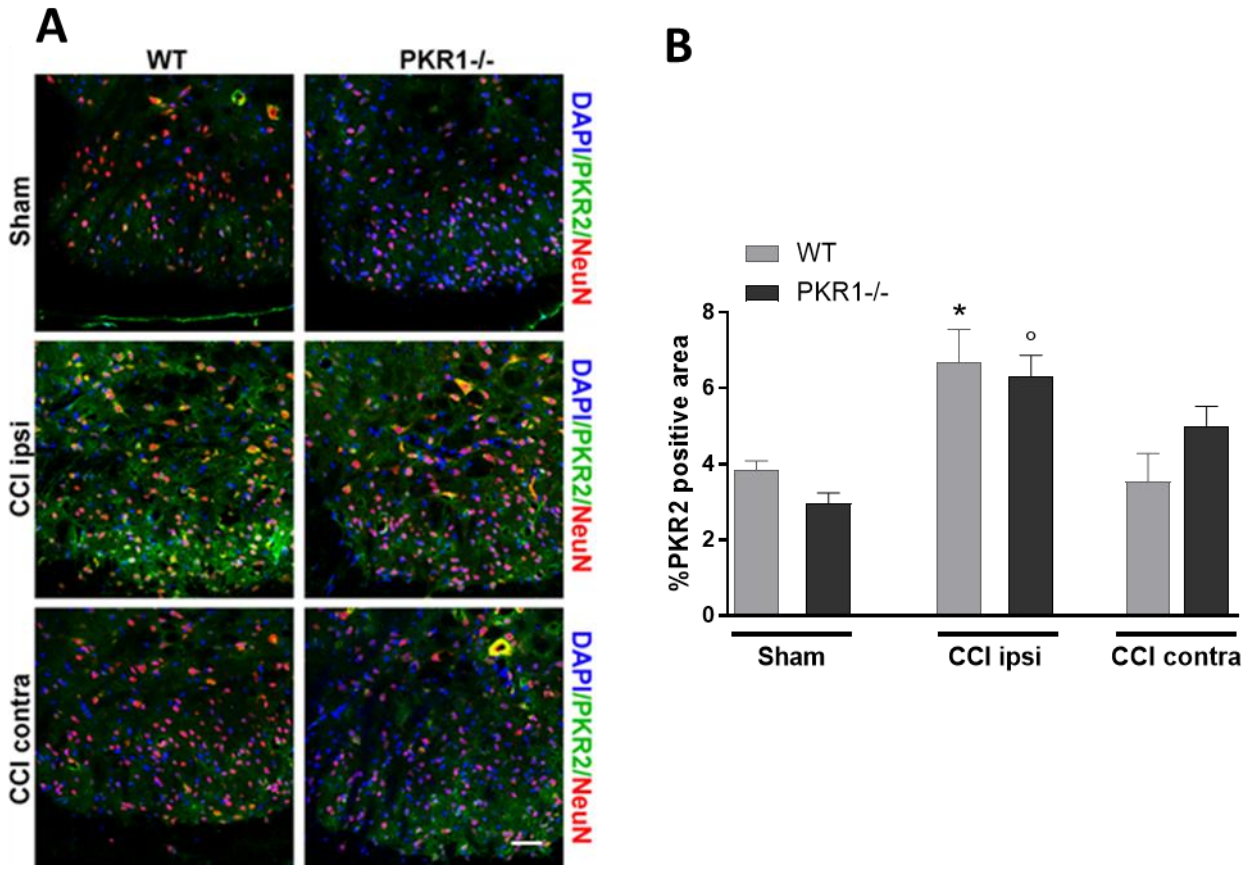

Fig 25. Following CCI surgery, WT and PKR1$^{-/-}$ mice show the same expression pattern of PKR2 in the spinal cord. A) PKR2 positive profiles (green) and NeuN (neuronal marker) positive profile (red) in WT and PKR1$^{-/-}$ mice. **B)** Quantification of PKR2 in the spinal cord. Sciatic nerve ligation induced a substantial increase of PKR2 signal 10 days after surgery in ipsilateral dorsal horn of both WT and PKR1$^{-/-}$ mice compared to sham animals. Very low expression of PKR2, instead, was detected in contralateral dorsal horn of all mice. Cell nuclei were counterstained with DAPI (blue). Scale bar: 50µm. *p < 0.05 vs WT sham; °p < 0.05 vs KO sham by one-way ANOVA with Tukey's.

3.2.4. Discussion

In this study, using a well-characterized model of chronic neuropathic pain resulting from constriction of the sciatic nerve in mice (Bennett and Xie, 1988) we investigated for the first time the modulation of PKR1 and PKR2.

Prokineticin system has been reported to be involved in a great number of physiological functions and thus its alterations underlie pathological mechanisms of several diseases. In particular, in the last few years, important results have been achieved in the comprehension on how this system can contribute to the development and maintenance of neuropathic pain states (Lattanzi et al., 2015; Maftei et al., 2014). Previous studies from our laboratory have demonstrated how mice lacking of prokineticin receptors show an impaired nociception and inflammatory pain sensation when compared with WT littermates (Negri et al., 2006). Knock out animals are an incredibly useful tool in pharmacological research, so, based on this literature, we decided to investigate the behavioural responses induced by peripheral nerve ligation in both $PKR1^{-/-}$ and $PKR2^{-/-}$ mice, to better characterize the contribution of each receptor in neuropathic pain development.

What came out from our very first experiment was unexpected and extremely interesting: almost immediately after CCI surgery, $PKR1^{-/-}$ mice developed a marked hypersensitivity to the contralateral paw, whereas both mechano-allodynia and thermal hyperalgesia threshold remain at baseline values in ipsilateral paw. $PKR2^{-/-}$ mice, instead, showed a "classical" development of neuropathic pain on ipsilateral side, at rates comparable with WT mice.

Modulation of prokineticin system in neuropathic pain conditions has been widely investigated in previous works from our lab (Lattanzi et al., 2015; Maftei et al., 2014; Negri and Maftei, 2018). However, no biochemical characterization of prokineticin system had been performed before on knock-out animals. Considering the puzzling behavioural results obtained, we decided to deeply investigate prokineticin system modulation in $PKR1^{-/-}$ mice. As expected, immunofluorescence analysis on lumbar spinal cord samples showed an overexpression of PROK2 in ipsilateral dorsal horn of WT mice. In $PKR1^{-/-}$ mice, instead, PROK2 resulted overexpressed both in ipsilateral and in contralateral portion of the spinal cord. This result, could partially explain what we observed during behavioural tests: whilst the fact that PROK2 is overexpressed on contralateral side could explain the contralateral neuropathy we measured, the fact that it is still elevated in ipsilateral side arises the question on why we can't register pain on ipsilateral side. Trying to find the answer, we also investigated PKR2 expression in the spinal cord. As previously reported (Maftei et al., 2014) the receptor results overexpressed following nerve ligation in ipsilateral dorsal horn of WT mice and

here, we confirmed this data. Once again, however, results in PKR1$^{-/-}$ mice surprised us: they showed the exact same pattern of expression of PKR2 observed in WT mice: high in ipsilateral side, but low in contralateral side.

Recently a spliced form of PKR2, named TM4-7, has been identified and characterized (Lattanzi et al., Neuropeptides, submitted). This alternative spliced form results overexpressed in correlation with inflammatory response. It could be possible that PROK2 overexpressed in contralateral side, could act on this spliced form instead of on wt form, and induce hypersensitivity on the contralateral side. No antibodies are available so far to target this truncated receptor. The one we have at the moment, binds the N-terminal region of PKR2, so it cannot recognize TM4-7 since, as its name indicates, it is lacking of the first 3 transmembrane regions, including N-terminal portion.

4. CONCLUSIONS

The data presented in this work throw new light on how we could intervene on such a huge medical need with major socioeconomic consequences as is the treatment of neuropathic pain.

A first approach is the blockade of pathways that contribute to neuroinflammation. Its overall accepted that activation of prokineticin system plays a critical role in development and maintenance of neuropathic pain. However, the latest data obtained on PKRs modulation increase our mechanistic understanding of the role of this system in chronic pain states. PKRs confirm its role as promising pharmacological target for the development of novel non-narcotic drugs to alleviate chronic pain.

The second approach is to empower anti-inflammatory and neuroprotective mechanisms: endogenous adenosine signals via A_3AR inhibit chronic neuropathic pain and A_3AR agonists have already advanced in clinical trials for non-pain indications showing a good safety profile. The findings presented in this work deeply investigate A_3AR agonists mechanism of action providing foundational and mechanistic rationale to support the clinical evaluation of these drugs also in neuropathic pain patients.

5. MATERIALS AND METHODS

5.1 Animals

Rag1 knock out (B6.129S7-Rag1tm1Mom/J), IL-10 knock out (B6.129P2-IL-10tm1Cgn/J) A_3AR knock out and wild-type (C57BL/6) male mice from The Jackson Laboratory (Bar Harbor, ME, USA) were used for the experiments on A3 Adenosine Receptor (A_3AR) agonists. Experiments were performed in accordance with International Association for the Study of Pain, U.S. National Institutes of Health guidelines on laboratory animal welfare, and St. Louis University Institutional Animal Care and Use Committee recommendations.

C57BL/6J wild-type and PKR1 knock out male mice from Lexicon Genetics (The Woodlands, TX, USA) were used for the experiments on Prokineticin system. Each protocol was approved by the Animal Care and Use Committee of the Italian Ministry of Health according to European Commission directives.

All animals weighed approximately 20-30 g at the moment of experiments and were housed 5/cage in a controlled environment (12-h light-dark cycles, 21 ± 2 °C, 50-60% humidity) with food and water available *ad libitum*. All efforts were made to minimize the number of animals used and their suffering. Experimenters were blinded to treatment conditions in all experiments.

5.2 Test Compounds

In this study, the highly selective A_3AR agonists, MRS5698, 2-(3,4-difluorophenylethynyl)-N6-(3-chlorobenzyl)-(N)-methanocarba-adenosine-5′-methyluronamide (Tosh et al., 2012) and MRS5980, (1S,2R,3S,4R,5S)-4-(2-((5-chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (Tosh et al., 2014) were used. Morphine was a kind gift from Mallinckrodt (St. Louis, MO, USA).

5.3 Procedures

5.3.1 CCI model of neuropathic pain

Chronic constriction injury to the sciatic nerve of the right hind leg in mice was performed under general anaesthesia using the well-characterized Bennett model (Bennett and Xie, 1988). Briefly,

mice were anesthetized either with 3% isoflurane/100% O2 inhalation and maintained on 2% isoflurane/100% O2 for the duration of surgery or with an intraperitoneal (i.p.) injection of a mixture of ketamine (60 mg/kg, Imalgene, Merial, Toulouse, France) and xylazine (10 mg/kg, Sigma-Aldrich, Saint Louis, MO, USA). The right thigh was shaved and a small incision (1–1.5 cm in length) was made in the middle of the lateral aspect of the right thigh to expose the sciatic nerve. The nerve was loosely ligated around the entire diameter at 3 distinct sites (spaced 1 mm apart) using silk sutures (6.0). The surgical site was then closed either a skin clip or with a silk suture (4.0). Mice were then allowed to recover in a heated cage until all reflexes were normalized. The injured and uninjured hind paws were named as ipsilateral and contralateral hind paw, respectively.

5.3.2 T cell isolation and adoptive transfer

Single-cell suspensions were obtained from spleens and lymph nodes of C57BL/6 WT, IL-10 knock out and A_3AR knock out mice by passing organs through 70µm strainers, after which cells were washed with PBS plus 0.1% bovine serum albumin. T-cell population was purified by negative selection. Briefly, T-cells were incubated with biotinylated antibodies against CD11b, CD11c, CD49b, B220, TER-119, CD4 and CD8a, all purchased from BioLegend, and they were negatively selected by autoMACS sorting. After MACS purification, T-cells were washed, counted and resuspended in PBS for intravenous injections (2 x 10^6/mouse). On Day 7 after CCI surgery, T cells or PBS were injected intravenously (i.v.) into the tail vein in a volume of 200µl. An aliquot of the sorted population was assessed for the purity check analysis: cells were labeled with anti-CD3-FITC or with anti-CD4-PeCy7 and the purity was determined by FlowCytometry.

5.3.3 Drugs injection

The highly selective A_3AR agonists, MRS5698 (1 mg/kg) and MRS5980 (1 mg/kg), were administered intraperitoneally (i.p.) on Day 8 after CCI surgery. Both compounds were dissolved in a solution of 10% of Dymethyl Sulfoxide (DMSO) in sterile saline. MRS5980 (3nmol/mouse) was administered also intrathecally (i.th.) to investigate its site of action. The drug also in this case was dissolved in a solution of 10% of DMSO in sterile saline. Morphine (3 mg/kg) was dissolved in sterile saline and administered i.p. on Day 9 after CCI surgery.

5.4 Behavioural Testing

5.4.1 Measurement of thermal hyperalgesia

For testing heat sensitivity, animals were put in plastic boxes and allowed 20-30 min for habituation before examination. Heat sensitivity was tested by radiant heat using Hargreaves apparatus (Ugo Basile, Italy) and expressed as paw withdrawal latency (PWL). The infrared emitter/detector was put directly underneath the plantar aspect of hindpaws. The radiant heat intensity was adjusted so that basal PWL is between 10 and 12 s with a cut-off of 20 s to prevent tissue damage.

5.4.2 Measurement of mechanical allodynia

Mechano-allodynia was measured after first acclimating the animals to elevated cages with a wire mesh floor for 15-20 min. The plantar aspect of hindpaws was probed 3 times with calibrated Von Frey filaments (Stoelting, Wood Dale, IL, USA; mice: 0.07–2.00 g) according to the "up-and-down" method (Dixon, 1980). Mechanical threshold was assessed 3 times at each time point to yield a mean value, reported as paw withdrawal threshold (PWT). The development of mechano-allodynia is evidenced by a significant ($P<0.05$) reduction in mechanical mean PWT at forces that failed to elicit withdrawal responses on D0 before CCI or drug/vehicle treatment.

5.5 Biochemical Assays

5.5.2 Immunofluorescence assay

For the study on A_3AR agonists: mice received an i.p. injection of ketamine (11 mg/kg)/xylazine (1.1 mg/kg) for deep anesthesia and were transcardially perfused with 1X PBS followed by 4% paraformaldehyde, pH 7.4. The lumbar portion of the spinal cord and lumbar DRGs (L4-L5) were harvested and post-fixed for 30 min at room temperature. Tissues were cryoprotected in a 30% sucrose solution at 4°C for 72 h and frozen in OCT. Samples were cryosectioned at 10 microns and blocked with 5% mouse, 5% goat, 1% BSA, 0.3 M glycine for 30 minutes at room temperature. A rat

anti-mouse CD4 (dilution 1:100; BioLegend) was incubated overnight at 4 °C in a humidity chamber. Following a series of rinses in PBS-T (Tween 20 0.25 %) and PBS, pH 7.4, the sections were incubated with an Alexa Fluor 568-conjugated goat anti-rat IgG (1:300; Life technologies). Prolong gold with DAPI was added and the slides were cover slipped. The images were acquired using Olympus FV1000 laser scanning confocal microscope.

For the study on prokineticin system: 10 days after CCI L4-L5 spinal cord was dissected from transcardially perfused mice (PBS followed by 4% paraformaldehyde), post-fixed in 4% PFA for 24 hours and cryoprotected in 30% sucrose solution. Tissues were embedded in cryostat medium, frozen and cut using a cryostat at 40 μm thick slices (free-floating). Prior to immunofluorescence staining sections were blocked with 3% normal donkey serum in 0.3% Triton X-100 for 30 min at room temperature and then incubated at 4°C for 48h with the following primary antibodies diluted in PBS-0.3% Triton X-100: rabbit polyclonal anti-PROK2 (1:200, AbCam, Cambridge, UK), rabbit polyclonal anti -PKR2 (1:200, Alomone labs, Jerusalem, Israel), mouse polyclonal anti-glialfibrillary acidic protein (GFAP) (1:400) and mouse monoclonal anti-neuronal nuclei (NeuN) (1:400, Immunological Sciences, Italy). After washing, sections were incubated for 2h at room temperature with anti-species IgG secondary antibodies coupled to Alexa Fluor®-488 or 555 (1:200, Immunological Sciences). Nuclei were stained with DAPI (1:500, Sigma Aldrich). Possible non-specific labeling of mouse secondary antibody was detected by using secondary antibody alone. The stained sections were examined at confocal laser scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany).

5.6 Statistical analysis

The data are expressed as mean ± SD for *n* animals and analysed by two-way repeated-measures ANOVA with Bonferroni post hoc test. Significant differences were defined as $p < 0.05$. GraphPad Prism 7 for Windows version 7.03.

6. REFERENCES:

1998. Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science (New York, N.Y.)* 282:2012-2018.

2004. Finishing the euchromatic sequence of the human genome. *Nature* 431:931-945.

Abou-Hamdan, M., M. Costanza, E. Fontana, M. Di Dario, S. Musio, C. Congiu, V. Onnis, R. Lattanzi, M. Radaelli, V. Martinelli, S. Salvadori, L. Negri, P.L. Poliani, C. Farina, G. Balboni, L. Steinman, and R. Pedotti. 2015. Critical role for prokineticin 2 in CNS autoimmunity. *Neurology(R) neuroimmunology & neuroinflammation* 2:e95.

Aharoni, R., B. Kayhan, R. Eilam, M. Sela, and R. Arnon. 2003. Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor in situ. *Proceedings of the National Academy of Sciences of the United States of America* 100:14157-14162.

Antonioli, L., M. Fornai, R. Colucci, N. Ghisu, M. Tuccori, O. Awwad, A. Bin, C. Zoppellaro, I. Castagliuolo, R.M. Gaion, M.C. Giron, and C. Blandizzi. 2010. Control of enteric neuromuscular functions by purinergic A(3) receptors in normal rat distal colon and experimental bowel inflammation. *British journal of pharmacology* 161:856-871.

Attal, N., and D. Bouhassira. 2015. Pharmacotherapy of neuropathic pain: which drugs, which treatment algorithms? *Pain* 156 Suppl 1:S104-114.

Attal, N., M. Lanteri-Minet, B. Laurent, J. Fermanian, and D. Bouhassira. 2011. The specific disease burden of neuropathic pain: results of a French nationwide survey. *Pain* 152:2836-2843.

Attramadal, H. 2009. Prokineticins and the heart: diverging actions elicited by signalling through prokineticin receptor-1 or -2. *Cardiovascular research* 81:3-4.

Austin, P.J., C.F. Kim, C.J. Perera, and G. Moalem-Taylor. 2012. Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis. *Pain* 153:1916-1931.

Baddack-Werncke, U., M. Busch-Dienstfertig, S. Gonzalez-Rodriguez, S.C. Maddila, J. Grobe, M. Lipp, C. Stein, and G. Muller. 2017. Cytotoxic T cells modulate inflammation and endogenous opioid analgesia in chronic arthritis. *Journal of neuroinflammation* 14:30.

Bagaitkar, J. 2014. Cellular dynamics of resolving inflammation. *Blood* 124:1701-1703.

Baharav, E., S. Bar-Yehuda, L. Madi, D. Silberman, L. Rath-Wolfson, M. Halpren, A. Ochaion, A. Weinberger, and P. Fishman. 2005. Antiinflammatory effect of A3 adenosine receptor agonists in murine autoimmune arthritis models. *The Journal of rheumatology* 32:469-476.

Bajetto, A., R. Bonavia, S. Barbero, and G. Schettini. 2002. Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. *Journal of neurochemistry* 82:1311-1329.

Balboni, G., I. Lazzari, C. Trapella, L. Negri, R. Lattanzi, E. Giannini, A. Nicotra, P. Melchiorri, S. Visentin, C.D. Nuccio, and S. Salvadori. 2008. Triazine compounds as antagonists at Bv8-prokineticin receptors. *Journal of medicinal chemistry* 51:7635-7639.

Ballarin, M., B.B. Fredholm, S. Ambrosio, and N. Mahy. 1991. Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta physiologica Scandinavica* 142:97-103.

Bar-Yehuda, S., D. Luger, A. Ochaion, S. Cohen, R. Patokaa, G. Zozulya, P.B. Silver, J.M. de Morales, R.R. Caspi, and P. Fishman. 2011. Inhibition of experimental auto-immune uveitis by the A3 adenosine receptor agonist CF101. *International journal of molecular medicine* 28:727-731.

Baron, R., A. Binder, and G. Wasner. 2010. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. *The Lancet. Neurology* 9:807-819.

Barrot, M. 2012. Tests and models of nociception and pain in rodents. *Neuroscience* 211:39-50.

Basbaum, A.I., D.M. Bautista, G. Scherrer, and D. Julius. 2009. Cellular and molecular mechanisms of pain. *Cell* 139:267-284.

Beavis, P.A., J. Stagg, P.K. Darcy, and M.J. Smyth. 2012. CD73: a potent suppressor of antitumor immune responses. *Trends in immunology* 33:231-237.

Bennett, G.J., and Y.K. Xie. 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107.

Boison, D. 2013. Adenosine kinase: exploitation for therapeutic gain. *Pharmacological reviews* 65:906-943.

Boison, D., J.F. Chen, and B.B. Fredholm. 2010. Adenosine signaling and function in glial cells. *Cell death and differentiation* 17:1071-1082.

Bonecchi, R., E. Galliera, E.M. Borroni, M.M. Corsi, M. Locati, and A. Mantovani. 2009. Chemokines and chemokine receptors: an overview. *Frontiers in bioscience (Landmark edition)* 14:540-551.

Bonica, J.J. 1953. The management of pain. *Philadelphia: Lea & Febiger*

Borea, P.A., K. Varani, F. Vincenzi, P.G. Baraldi, M.A. Tabrizi, S. Merighi, and S. Gessi. 2015. The A3 adenosine receptor: history and perspectives. *Pharmacological reviews* 67:74-102.

Bouma, M.G., T.M. Jeunhomme, D.L. Boyle, M.A. Dentener, N.N. Voitenok, F.A. van den Wildenberg, and W.A. Buurman. 1997. Adenosine inhibits neutrophil degranulation in

activated human whole blood: involvement of adenosine A2 and A3 receptors. *Journal of immunology (Baltimore, Md. : 1950)* 158:5400-5408.

Boyman, O., and J. Sprent. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nature reviews. Immunology* 12:180-190.

Brand, A., Z. Vissiennon, D. Eschke, and K. Nieber. 2001. Adenosine A(1) and A(3) receptors mediate inhibition of synaptic transmission in rat cortical neurons. *Neuropharmacology* 40:85-95.

Breivik, H., B. Collett, V. Ventafridda, R. Cohen, and D. Gallacher. 2006. Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *European journal of pain (London, England)* 10:287-333.

Broussas, M., P. Cornillet-Lefebvre, G. Potron, and P. Nguyen. 2002. Adenosine inhibits tissue factor expression by LPS-stimulated human monocytes: involvement of the A3 adenosine receptor. *Thrombosis and haemostasis* 88:123-130.

Bullock, C.M., J.D. Li, and Q.Y. Zhou. 2004. Structural determinants required for the bioactivities of prokineticins and identification of prokineticin receptor antagonists. *Molecular pharmacology* 65:582-588.

Burnett, L.A., E.M. Blais, J.D. Unadkat, B. Hille, S.L. Tilley, and D.F. Babcock. 2010. Testicular expression of Adora3i2 in Adora3 knockout mice reveals a role of mouse A3Ri2 and human A3Ri3 adenosine receptors in sperm. *The Journal of biological chemistry* 285:33662-33670.

Calvo, M., J.M. Dawes, and D.L. Bennett. 2012. The role of the immune system in the generation of neuropathic pain. *The Lancet. Neurology* 11:629-642.

Cao, L., and J.A. DeLeo. 2008. CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *European journal of immunology* 38:448-458.

Cartier, L., O. Hartley, M. Dubois-Dauphin, and K.H. Krause. 2005. Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain research. Brain research reviews* 48:16-42.

Challa, S.R. 2015. Surgical animal models of neuropathic pain: Pros and Cons. *The International journal of neuroscience* 125:170-174.

Chavan, S.S., V.A. Pavlov, and K.J. Tracey. 2017. Mechanisms and Therapeutic Relevance of Neuro-immune Communication. *Immunity* 46:927-942.

Chen, B., L. Yu, J. Wang, C. Li, K. Zhao, and H. Zhang. 2016. Involvement of Prokineticin 2 and Prokineticin Receptor 1 in Lipopolysaccharide-Induced Testitis in Rats. *Inflammation* 39:534-542.

Chen, J., C. Kuei, S. Sutton, S. Wilson, J. Yu, F. Kamme, C. Mazur, T. Lovenberg, and C. Liu. 2005. Identification and pharmacological characterization of prokineticin 2 beta as a selective ligand for prokineticin receptor 1. *Molecular pharmacology* 67:2070-2076.

Chen, M., and J.L. Manley. 2009. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nature reviews. Molecular cell biology* 10:741-754.

Chen, Z., K. Janes, C. Chen, T. Doyle, L. Bryant, D.K. Tosh, K.A. Jacobson, and D. Salvemini. 2012. Controlling murine and rat chronic pain through A3 adenosine receptor activation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 26:1855-1865.

Cheng, M.Y., C.M. Bullock, C. Li, A.G. Lee, J.C. Bermak, J. Belluzzi, D.R. Weaver, F.M. Leslie, and Q.Y. Zhou. 2002. Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* 417:405-410.

Cheng, M.Y., F.M. Leslie, and Q.Y. Zhou. 2006. Expression of prokineticins and their receptors in the adult mouse brain. *The Journal of comparative neurology* 498:796-809.

Cinalli, A.R., J.F. Guarracino, V. Fernandez, L.I. Roquel, and A.S. Losavio. 2013. Inosine induces presynaptic inhibition of acetylcholine release by activation of A3 adenosine receptors at the mouse neuromuscular junction. *British journal of pharmacology* 169:1810-1823.

Cobos, E.J., N. Ghasemlou, D. Araldi, D. Segal, K. Duong, and C.J. Woolf. 2012. Inflammation-induced decrease in voluntary wheel running in mice: a nonreflexive test for evaluating inflammatory pain and analgesia. *Pain* 153:876-884.

Cohen, S., S.M. Stemmer, G. Zozulya, A. Ochaion, R. Patoka, F. Barer, S. Bar-Yehuda, L. Rath-Wolfson, K.A. Jacobson, and P. Fishman. 2011. CF102 an A3 adenosine receptor agonist mediates anti-tumor and anti-inflammatory effects in the liver. *Journal of cellular physiology* 226:2438-2447.

Cook, I.H., J. Evans, D. Maldonado-Perez, H.O. Critchley, K.J. Sales, and H.N. Jabbour. 2010. Prokineticin-1 (PROK1) modulates interleukin (IL)-11 expression via prokineticin receptor 1 (PROKR1) and the calcineurin/NFAT signalling pathway. *Molecular human reproduction* 16:158-169.

Corriden, R., T. Self, K. Akong-Moore, V. Nizet, B. Kellam, S.J. Briddon, and S.J. Hill. 2013. Adenosine-A3 receptors in neutrophil microdomains promote the formation of bacteria-tethering cytonemes. *EMBO reports* 14:726-732.

Costigan, M., A. Moss, A. Latremoliere, C. Johnston, M. Verma-Gandhu, T.A. Herbert, L. Barrett, G.J. Brenner, D. Vardeh, C.J. Woolf, and M. Fitzgerald. 2009. T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity.

The Journal of neuroscience : the official journal of the Society for Neuroscience 29:14415-14422.

Cunha, R.A. 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochemistry international* 38:107-125.

David, M., D.K. Gospodinov, N. Gheorghe, G.S. Mateev, M.V. Rusinova, E. Hristakieva, L.G. Solovastru, R.V. Patel, C. Giurcaneanu, M.C. Hitova, A.I. Purcaru, B. Horia, Tsingov, II, R.K. Yankova, M.I. Kadurina, M. Ramon, M. Rotaru, O. Simionescu, V. Benea, Z.V. Demerdjieva, M.R. Cosgarea, H.S. Morariu, Z. Michael, P. Cristodor, C. Nica, M.H. Silverman, D.R. Bristol, Z. Harpaz, M. Farbstein, S. Cohen, and P. Fishman. 2016. Treatment of Plaque-Type Psoriasis With Oral CF101: Data from a Phase II/III Multicenter, Randomized, Controlled Trial. *Journal of drugs in dermatology : JDD* 15:931-938.

Davis, K.D., H. Flor, H.T. Greely, G.D. Iannetti, S. Mackey, M. Ploner, A. Pustilnik, I. Tracey, R.D. Treede, and T.D. Wager. 2017. Brain imaging tests for chronic pain: medical, legal and ethical issues and recommendations. *Nature reviews. Neurology* 13:624-638.

De Felice, M., P. Melchiorri, M.H. Ossipov, T.W. Vanderah, F. Porreca, and L. Negri. 2012. Mechanisms of Bv8-induced biphasic hyperalgesia: increased excitatory transmitter release and expression. *Neuroscience letters* 521:40-45.

de Mendonca, A., and J.A. Ribeiro. 1997. Adenosine and neuronal plasticity. *Life sciences* 60:245-251.

de Novellis, V., L. Negri, R. Lattanzi, F. Rossi, E. Palazzo, I. Marabese, E. Giannini, D. Vita, P. Melchiorri, and S. Maione. 2007. The prokineticin receptor agonist Bv8 increases GABA release in the periaqueductal grey and modifies RVM cell activities and thermoceptive reflexes in the rat. *The European journal of neuroscience* 26:3068-3078.

Decosterd, I., and C.J. Woolf. 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87:149-158.

Dib-Hajj, S.D., J.A. Black, and S.G. Waxman. 2009. Voltage-gated sodium channels: therapeutic targets for pain. *Pain medicine (Malden, Mass.)* 10:1260-1269.

Dixon, A.K., A.K. Gubitz, D.J. Sirinathsinghji, P.J. Richardson, and T.C. Freeman. 1996. Tissue distribution of adenosine receptor mRNAs in the rat. *British journal of pharmacology* 118:1461-1468.

Dixon, W.J. 1980. Efficient analysis of experimental observations. *Annual review of pharmacology and toxicology* 20:441-462.

Dorsch, M., Y. Qiu, D. Soler, N. Frank, T. Duong, A. Goodearl, S. O'Neil, J. Lora, and C.C. Fraser. 2005. PK1/EG-VEGF induces monocyte differentiation and activation. *Journal of leukocyte biology* 78:426-434.

Dosch, M., J. Gerber, F. Jebbawi, and G. Beldi. 2018. Mechanisms of ATP Release by Inflammatory Cells. *International journal of molecular sciences* 19:

Draleau, K., S. Maddula, A. Slaiby, N. Nutile-McMenemy, J. De Leo, and L. Cao. 2014. Phenotypic Identification of Spinal Cord-Infiltrating CD4(+) T Lymphocytes in a Murine Model of Neuropathic Pain. *Journal of pain & relief* Suppl 3:003.

Dubin, A.E., and A. Patapoutian. 2010. Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation* 120:3760-3772.

Fang, Z.Z., and F.J. Gonzalez. 2014. LC-MS-based metabolomics: an update. *Archives of toxicology* 88:1491-1502.

Fang, Z.Z., D.K. Tosh, N. Tanaka, H. Wang, K.W. Krausz, R. O'Connor, K.A. Jacobson, and F.J. Gonzalez. 2015. Metabolic mapping of A3 adenosine receptor agonist MRS5980. *Biochemical pharmacology* 97:215-223.

Finnerup, N.B., N. Attal, S. Haroutounian, E. McNicol, R. Baron, R.H. Dworkin, I. Gilron, M. Haanpaa, P. Hansson, T.S. Jensen, P.R. Kamerman, K. Lund, A. Moore, S.N. Raja, A.S. Rice, M. Rowbotham, E. Sena, P. Siddall, B.H. Smith, and M. Wallace. 2015. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *The Lancet. Neurology* 14:162-173.

Finnerup, N.B., S.H. Sindrup, and T.S. Jensen. 2010. The evidence for pharmacological treatment of neuropathic pain. *Pain* 150:573-581.

Fishman, P., S. Bar-Yehuda, E. Ardon, L. Rath-Wolfson, F. Barrer, A. Ochaion, and L. Madi. 2003. Targeting the A3 adenosine receptor for cancer therapy: inhibition of prostate carcinoma cell growth by A3AR agonist. *Anticancer research* 23:2077-2083.

Fishman, P., S. Bar-Yehuda, F. Barer, L. Madi, A.S. Multani, and S. Pathak. 2001. The A3 adenosine receptor as a new target for cancer therapy and chemoprotection. *Experimental cell research* 269:230-236.

Fishman, P., S. Bar-Yehuda, B.T. Liang, and K.A. Jacobson. 2012. Pharmacological and therapeutic effects of A3 adenosine receptor agonists. *Drug discovery today* 17:359-366.

Fishman, P., S. Bar-Yehuda, L. Madi, L. Rath-Wolfson, A. Ochaion, S. Cohen, and E. Baharav. 2006. The PI3K-NF-kappaB signal transduction pathway is involved in mediating the anti-inflammatory effect of IB-MECA in adjuvant-induced arthritis. *Arthritis research & therapy* 8:R33.

Florea, L., V. Di Francesco, J. Miller, R. Turner, A. Yao, M. Harris, B. Walenz, C. Mobarry, G.V. Merkulov, R. Charlab, I. Dew, Z. Deng, S. Istrail, P. Li, and G. Sutton. 2005. Gene and alternative splicing annotation with AIR. *Genome research* 15:54-66.

Ford, A., A. Castonguay, M. Cottet, J.W. Little, Z. Chen, A.M. Symons-Liguori, T. Doyle, T.M. Egan, T.W. Vanderah, Y. De Koninck, D.K. Tosh, K.A. Jacobson, and D. Salvemini. 2015. Engagement of the GABA to KCC2 signaling pathway contributes to the analgesic effects of A3AR agonists in neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 35:6057-6067.

Fossetta, J., J. Jackson, G. Deno, X. Fan, X.K. Du, L. Bober, A. Soude-Bermejo, O. de Bouteiller, C. Caux, C. Lunn, D. Lundell, and R.K. Palmer. 2003. Pharmacological analysis of calcium responses mediated by the human A3 adenosine receptor in monocyte-derived dendritic cells and recombinant cells. *Molecular pharmacology* 63:342-350.

Foster, J.R. 2001. The functions of cytokines and their uses in toxicology. *International journal of experimental pathology* 82:171-192.

Franchi, S., E. Giannini, D. Lattuada, R. Lattanzi, H. Tian, P. Melchiorri, L. Negri, A.E. Panerai, and P. Sacerdote. 2008. The prokineticin receptor agonist Bv8 decreases IL-10 and IL-4 production in mice splenocytes by activating prokineticin receptor-1. *BMC immunology* 9:60.

Fredholm, B.B. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death and differentiation* 14:1315-1323.

Fredholm, B.B., I.J. AP, K.A. Jacobson, J. Linden, and C.E. Muller. 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacological reviews* 63:1-34.

Gasser, A., S. Brogi, K. Urayama, T. Nishi, H. Kurose, A. Tafi, N. Ribeiro, L. Desaubry, and C.G. Nebigil. 2015. Discovery and cardioprotective effects of the first non-Peptide agonists of the G protein-coupled prokineticin receptor-1. *PloS one* 10:e0121027.

Gattlen, C., C.B. Clarke, N. Piller, G. Kirschmann, M. Pertin, I. Decosterd, R.D. Gosselin, and M.R. Suter. 2016. Spinal Cord T-Cell Infiltration in the Rat Spared Nerve Injury Model: A Time Course Study. *International journal of molecular sciences* 17:352.

Gessi, S., S. Merighi, A. Stefanelli, D. Fazzi, K. Varani, and P.A. Borea. 2013. A(1) and A(3) adenosine receptors inhibit LPS-induced hypoxia-inducible factor-1 accumulation in murine astrocytes. *Pharmacological research* 76:157-170.

Gessi, S., K. Varani, S. Merighi, E. Cattabriga, A. Avitabile, R. Gavioli, C. Fortini, E. Leung, S. Mac Lennan, and P.A. Borea. 2004. Expression of A3 adenosine receptors in human lymphocytes: up-regulation in T cell activation. *Molecular pharmacology* 65:711-719.

Giannini, E., R. Lattanzi, A. Nicotra, A.F. Campese, P. Grazioli, I. Screpanti, G. Balboni, S. Salvadori, P. Sacerdote, and L. Negri. 2009. The chemokine Bv8/prokineticin 2 is up-regulated in inflammatory granulocytes and modulates inflammatory pain. *Proceedings of the National Academy of Sciences of the United States of America* 106:14646-14651.

Gilbert, W. 1978. Why genes in pieces? *Nature* 271:501.

Goldberg, D.S., and S.J. McGee. 2011. Pain as a global public health priority. *BMC public health* 11:770.

Gonzalez, H., D. Elgueta, A. Montoya, and R. Pacheco. 2014. Neuroimmune regulation of microglial activity involved in neuroinflammation and neurodegenerative diseases. *Journal of neuroimmunology* 274:1-13.

Grace, P.M., M.R. Hutchinson, S.F. Maier, and L.R. Watkins. 2014. Pathological pain and the neuroimmune interface. *Nature reviews. Immunology* 14:217-231.

Grace, P.M., P.E. Rolan, and M.R. Hutchinson. 2011. Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. *Brain, behavior, and immunity* 25:1322-1332.

Gregoire, S., V. Michaud, E. Chapuy, A. Eschalier, and D. Ardid. 2012. Study of emotional and cognitive impairments in mononeuropathic rats: effect of duloxetine and gabapentin. *Pain* 153:1657-1663.

Gross, P.M., M. Kadekaro, L. Sokoloff, H.H. Holcomb, and J.M. Saavedra. 1985. Alterations of local cerebral glucose utilization during chronic dehydration in rats. *Brain research* 330:329-336.

Guida, F., R. Lattanzi, S. Boccella, D. Maftei, R. Romano, V. Marconi, G. Balboni, S. Salvadori, M.A. Scafuro, V. de Novellis, L. Negri, S. Maione, and L. Luongo. 2015. PC1, a non-peptide PKR1-preferring antagonist, reduces pain behavior and spinal neuronal sensitization in neuropathic mice. *Pharmacological research* 91:36-46.

Guzman, J., J.G. Yu, Z. Suntres, A. Bozarov, H. Cooke, N. Javed, H. Auer, J. Palatini, H.H. Hassanain, A.J. Cardounel, A. Javed, I. Grants, J.E. Wunderlich, and F.L. Christofi. 2006. ADOA3R as a therapeutic target in experimental colitis: proof by validated high-density oligonucleotide microarray analysis. *Inflammatory bowel diseases* 12:766-789.

Haroutounian, S., L. Nikolajsen, T.F. Bendtsen, N.B. Finnerup, A.D. Kristensen, J.B. Hasselstrom, and T.S. Jensen. 2014. Primary afferent input critical for maintaining spontaneous pain in peripheral neuropathy. *Pain* 155:1272-1279.

Hasko, G., and B. Cronstein. 2013. Regulation of inflammation by adenosine. *Frontiers in immunology* 4:85.

Hasko, G., J. Linden, B. Cronstein, and P. Pacher. 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nature reviews. Drug discovery* 7:759-770.

Hayashida, M., K. Fukuda, and A. Fukunaga. 2005. Clinical application of adenosine and ATP for pain control. *Journal of anesthesia* 19:225-235.

He, Z., Q. Guo, M. Xiao, C. He, and W. Zou. 2013. Intrathecal lentivirus-mediated transfer of interleukin-10 attenuates chronic constriction injury-induced neuropathic pain through modulation of spinal high-mobility group box 1 in rats. *Pain physician* 16:E615-625.

Hillebrand, J.J., D. de Wied, and R.A. Adan. 2002. Neuropeptides, food intake and body weight regulation: a hypothalamic focus. *Peptides* 23:2283-2306.

Howard, M., and A. O'Garra. 1992. Biological properties of interleukin 10. *Immunology today* 13:198-200.

Hu, P., and E.M. McLachlan. 2002. Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat. *Neuroscience* 112:23-38.

Hu, W.P., C. Zhang, J.D. Li, Z.D. Luo, S. Amadesi, N. Bunnett, and Q.Y. Zhou. 2006. Impaired pain sensation in mice lacking prokineticin 2. *Molecular pain* 2:35.

Ito, H., K. Noda, K. Yoshida, K. Otani, M. Yoshiga, Y. Oto, S. Saito, and D. Kurosaka. 2016. Prokineticin 2 antagonist, PKRA7 suppresses arthritis in mice with collagen-induced arthritis. *BMC musculoskeletal disorders* 17:387.

Jacobson, K.A., and Z.G. Gao. 2006. Adenosine receptors as therapeutic targets. *Nature reviews. Drug discovery* 5:247-264.

Jacobson, K.A., Z.G. Gao, A. Goblyos, and A.P. Ijzerman. 2011. Allosteric modulation of purine and pyrimidine receptors. *Advances in pharmacology (San Diego, Calif.)* 61:187-220.

Jacobson, K.A., X. Ji, A.H. Li, N. Melman, M.A. Siddiqui, K.J. Shin, V.E. Marquez, and R.G. Ravi. 2000. Methanocarba analogues of purine nucleosides as potent and selective adenosine receptor agonists. *Journal of medicinal chemistry* 43:2196-2203.

Janes, K., E. Esposito, T. Doyle, S. Cuzzocrea, D.K. Tosh, K.A. Jacobson, and D. Salvemini. 2014. A3 adenosine receptor agonist prevents the development of paclitaxel-induced neuropathic pain by modulating spinal glial-restricted redox-dependent signaling pathways. *Pain* 155:2560-2567.

Janes, K., A.M. Symons-Liguori, K.A. Jacobson, and D. Salvemini. 2016. Identification of A3 adenosine receptor agonists as novel non-narcotic analgesics. *British journal of pharmacology* 173:1253-1267.

Janes, K., C. Wahlman, J.W. Little, T. Doyle, D.K. Tosh, K.A. Jacobson, and D. Salvemini. 2015. Spinal neuroimmune activation is independent of T-cell infiltration and attenuated by A3

adenosine receptor agonists in a model of oxaliplatin-induced peripheral neuropathy. *Brain, behavior, and immunity* 44:91-99.

Jensen, T.S., R. Baron, M. Haanpaa, E. Kalso, J.D. Loeser, A.S. Rice, and R.D. Treede. 2011. A new definition of neuropathic pain. *Pain* 152:2204-2205.

Jensen, T.S., and N.B. Finnerup. 2014. Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *The Lancet. Neurology* 13:924-935.

Ji, R.R., A. Chamessian, and Y.Q. Zhang. 2016. Pain regulation by non-neuronal cells and inflammation. *Science (New York, N.Y.)* 354:572-577.

Ji, R.R., Z.Z. Xu, and Y.J. Gao. 2014. Emerging targets in neuroinflammation-driven chronic pain. *Nature reviews. Drug discovery* 13:533-548.

Kalra, S.P., M.G. Dube, S. Pu, B. Xu, T.L. Horvath, and P.S. Kalra. 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine reviews* 20:68-100.

Kaser, A., M. Winklmayr, G. Lepperdinger, and G. Kreil. 2003. The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO reports* 4:469-473.

Kelemen, O., P. Convertini, Z. Zhang, Y. Wen, M. Shen, M. Falaleeva, and S. Stamm. 2013. Function of alternative splicing. *Gene* 514:1-30.

Kiguchi, N., D. Kobayashi, F. Saika, S. Matsuzaki, and S. Kishioka. 2017. Pharmacological Regulation of Neuropathic Pain Driven by Inflammatory Macrophages. *International journal of molecular sciences* 18:

Kiguchi, N., Y. Kobayashi, and S. Kishioka. 2012. Chemokines and cytokines in neuroinflammation leading to neuropathic pain. *Current opinion in pharmacology* 12:55-61.

Kiguchi, N., Y. Kobayashi, T. Maeda, F. Saika, and S. Kishioka. 2010. CC-chemokine MIP-1alpha in the spinal cord contributes to nerve injury-induced neuropathic pain. *Neuroscience letters* 484:17-21.

Kim, H.O., X.D. Ji, S.M. Siddiqi, M.E. Olah, G.L. Stiles, and K.A. Jacobson. 1994. 2-Substitution of N6-benzyladenosine-5'-uronamides enhances selectivity for A3 adenosine receptors. *Journal of medicinal chemistry* 37:3614-3621.

Kim, S.H., and J.M. Chung. 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50:355-363.

Kishi, T., T. Kitajima, T. Tsunoka, T. Okumura, M. Ikeda, T. Okochi, Y. Kinoshita, K. Kawashima, Y. Yamanouchi, N. Ozaki, and N. Iwata. 2009. Possible association of prokineticin 2 receptor gene (PROKR2) with mood disorders in the Japanese population. *Neuromolecular medicine* 11:114-122.

Kohno, Y., X. Ji, S.D. Mawhorter, M. Koshiba, and K.A. Jacobson. 1996. Activation of A3 adenosine receptors on human eosinophils elevates intracellular calcium. *Blood* 88:3569-3574.

Koleva, D., I. Krulichova, G. Bertolini, V. Caimi, and L. Garattini. 2005. Pain in primary care: an Italian survey. *European journal of public health* 15:475-479.

Kronfol, Z., and D.G. Remick. 2000. Cytokines and the brain: implications for clinical psychiatry. *The American journal of psychiatry* 157:683-694.

Krukowski, K., N. Eijkelkamp, G. Laumet, C.E. Hack, Y. Li, P.M. Dougherty, C.J. Heijnen, and A. Kavelaars. 2016. CD8+ T Cells and Endogenous IL-10 Are Required for Resolution of Chemotherapy-Induced Neuropathic Pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 36:11074-11083.

Kurejova, M., U. Nattenmuller, U. Hildebrandt, D. Selvaraj, S. Stosser, and R. Kuner. 2010. An improved behavioural assay demonstrates that ultrasound vocalizations constitute a reliable indicator of chronic cancer pain and neuropathic pain. *Molecular pain* 6:18.

LaGraize, S.C., C.J. Labuda, M.A. Rutledge, R.L. Jackson, and P.N. Fuchs. 2004. Differential effect of anterior cingulate cortex lesion on mechanical hypersensitivity and escape/avoidance behavior in an animal model of neuropathic pain. *Experimental neurology* 188:139-148.

Latini, S., and F. Pedata. 2001. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *Journal of neurochemistry* 79:463-484.

Lattanzi, R., C. Congiu, V. Onnis, A. Deplano, S. Salvadori, V. Marconi, D. Maftei, A. Francioso, S. Ambrosio, I. Casella, T. Costa, G. Caltalbiano, M.T. Matsoukas, G. Balboni, and L. Negri. 2014. Halogenated triazinediones behave as antagonists of PKR1: in vitro and in vivo pharmacological characterization. *Int J Pharm Sci and Res* 5:5064-5072.

Lattanzi, R., D. Maftei, V. Marconi, F. Florenzano, S. Franchi, E. Borsani, L.F. Rodella, G. Balboni, S. Salvadori, P. Sacerdote, and L. Negri. 2015. Prokineticin 2 upregulation in the peripheral nervous system has a major role in triggering and maintaining neuropathic pain in the chronic constriction injury model. *BioMed research international* 2015:301292.

Lattanzi, R., P. Sacerdote, S. Franchi, M. Canestrelli, R. Miele, D. Barra, S. Visentin, C. DeNuccio, F. Porreca, M. De Felice, F. Guida, L. Luongo, V. de Novellis, S. Maione, and L. Negri. 2012. Pharmacological activity of a Bv8 analogue modified in position 24. *British journal of pharmacology* 166:950-963.

Lau, D., S.E. Harte, T.J. Morrow, S. Wang, M. Mata, and D.J. Fink. 2012. Herpes simplex virus vector-mediated expression of interleukin-10 reduces below-level central neuropathic pain after spinal cord injury. *Neurorehabilitation and neural repair* 26:889-897.

Laumet, G., J.D. Edralin, A.C. Chiang, R. Dantzer, C.J. Heijnen, and A. Kavelaars. 2018. Resolution of inflammation-induced depression requires T lymphocytes and endogenous brain interleukin-10 signaling. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*

LeCouter, J., and N. Ferrara. 2003. EG-VEGF and Bv8. a novel family of tissue-selective mediators of angiogenesis, endothelial phenotype, and function. *Trends in cardiovascular medicine* 13:276-282.

LeCouter, J., J. Kowalski, J. Foster, P. Hass, Z. Zhang, L. Dillard-Telm, G. Frantz, L. Rangell, L. DeGuzman, G.A. Keller, F. Peale, A. Gurney, K.J. Hillan, and N. Ferrara. 2001. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* 412:877-884.

LeCouter, J., R. Lin, G. Frantz, Z. Zhang, K. Hillan, and N. Ferrara. 2003a. Mouse endocrine gland-derived vascular endothelial growth factor: a distinct expression pattern from its human ortholog suggests different roles as a regulator of organ-specific angiogenesis. *Endocrinology* 144:2606-2616.

LeCouter, J., R. Lin, M. Tejada, G. Frantz, F. Peale, K.J. Hillan, and N. Ferrara. 2003b. The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 100:2685-2690.

Ledeboer, A., J.J. Breve, A. Wierinckx, S. van der Jagt, A.F. Bristow, J.E. Leysen, F.J. Tilders, and A.M. Van Dam. 2002. Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells. *The European journal of neuroscience* 16:1175-1185.

Lee, B.S., I.G. Jun, S.H. Kim, and J.Y. Park. 2013. Intrathecal gabapentin increases interleukin-10 expression and inhibits pro-inflammatory cytokine in a rat model of neuropathic pain. *Journal of Korean medical science* 28:308-314.

Lees, J.G., S.S. Duffy, C.J. Perera, and G. Moalem-Taylor. 2015. Depletion of Foxp3+ regulatory T cells increases severity of mechanical allodynia and significantly alters systemic cytokine levels following peripheral nerve injury. *Cytokine* 71:207-214.

Leger, T., J. Grist, F. D'Acquisto, A.K. Clark, and M. Malcangio. 2011. Glatiramer acetate attenuates neuropathic allodynia through modulation of adaptive immune cells. *Journal of neuroimmunology* 234:19-26.

Levit, A., T. Yarnitzky, A. Wiener, R. Meidan, and M.Y. Niv. 2011. Modeling of human prokineticin receptors: interactions with novel small-molecule binders and potential off-target drugs. *PloS one* 6:e27990.

Li, A.H., S. Moro, N. Melman, X.D. Ji, and K.A. Jacobson. 1998. Structure-activity relationships and molecular modeling of 3, 5-diacyl-2,4-dialkylpyridine derivatives as selective A3 adenosine receptor antagonists. *Journal of medicinal chemistry* 41:3186-3201.

Li, J.D., W.P. Hu, and Q.Y. Zhou. 2009. Disruption of the circadian output molecule prokineticin 2 results in anxiolytic and antidepressant-like effects in mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 34:367-373.

Li, M., C.M. Bullock, D.J. Knauer, F.J. Ehlert, and Q.Y. Zhou. 2001. Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Molecular pharmacology* 59:692-698.

Libri, D.V., G. Kleinau, V. Vezzoli, M. Busnelli, F. Guizzardi, A.A. Sinisi, A.I. Pincelli, A. Mancini, G. Russo, P. Beck-Peccoz, S. Loche, C. Crivellaro, M. Maghnie, C. Krausz, L. Persani, and M. Bonomi. 2014. Germline prokineticin receptor 2 (PROKR2) variants associated with central hypogonadism cause differental modulation of distinct intracellular pathways. *The Journal of clinical endocrinology and metabolism* 99:E458-463.

Lin, D.C., C.M. Bullock, F.J. Ehlert, J.L. Chen, H. Tian, and Q.Y. Zhou. 2002a. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *The Journal of biological chemistry* 277:19276-19280.

Lin, R., J. LeCouter, J. Kowalski, and N. Ferrara. 2002b. Characterization of Endocrine Gland-derived Vascular Endothelial Growth Factor Signaling in Adrenal Cortex Capillary Endothelial Cells. *Journal of Biological Chemistry* 277:8724-8729.

Linden, J. 1994. Cloned adenosine A3 receptors: pharmacological properties, species differences and receptor functions. *Trends in pharmacological sciences* 15:298-306.

Little, J.W., A. Ford, A.M. Symons-Liguori, Z. Chen, K. Janes, T. Doyle, J. Xie, L. Luongo, D.K. Tosh, S. Maione, K. Bannister, A.H. Dickenson, T.W. Vanderah, F. Porreca, K.A. Jacobson, and D. Salvemini. 2015. Endogenous adenosine A3 receptor activation selectively alleviates persistent pain states. *Brain : a journal of neurology* 138:28-35.

Liu, M., and J.N. Wood. 2011. The roles of sodium channels in nociception: implications for mechanisms of neuropathic pain. *Pain medicine (Malden, Mass.)* 12 Suppl 3:S93-99.

Liu, X.L., R. Zhou, Q.Q. Pan, X.L. Jia, W.N. Gao, J. Wu, J. Lin, and J.F. Chen. 2010. Genetic inactivation of the adenosine A2A receptor attenuates pathologic but not developmental angiogenesis in the mouse retina. *Investigative ophthalmology & visual science* 51:6625-6632.

Loeser, J.D., and R.D. Treede. 2008. The Kyoto protocol of IASP Basic Pain Terminology. *Pain* 137:473-477.

Lopes, L.V., N. Rebola, P.C. Pinheiro, P.J. Richardson, C.R. Oliveira, and R.A. Cunha. 2003. Adenosine A3 receptors are located in neurons of the rat hippocampus. *Neuroreport* 14:1645-1648.

Loram, L.C., J.A. Harrison, E.M. Sloane, M.R. Hutchinson, P. Sholar, F.R. Taylor, D. Berkelhammer, B.D. Coats, S. Poole, E.D. Milligan, S.F. Maier, J. Rieger, and L.R. Watkins. 2009. Enduring reversal of neuropathic pain by a single intrathecal injection of adenosine 2A receptor agonists: a novel therapy for neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29:14015-14025.

Madi, L., A. Ochaion, L. Rath-Wolfson, S. Bar-Yehuda, A. Erlanger, G. Ohana, A. Harish, O. Merimski, F. Barer, and P. Fishman. 2004. The A3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10:4472-4479.

Maftei, D., V. Marconi, F. Florenzano, L.A. Giancotti, M. Castelli, S. Moretti, E. Borsani, L.F. Rodella, G. Balboni, L. Luongo, S. Maione, P. Sacerdote, L. Negri, and R. Lattanzi. 2014. Controlling the activation of the Bv8/prokineticin system reduces neuroinflammation and abolishes thermal and tactile hyperalgesia in neuropathic animals. *British journal of pharmacology* 171:4850-4865.

Mailliard, R.B., S. Egawa, Q. Cai, A. Kalinska, S.N. Bykovskaya, M.T. Lotze, M.L. Kapsenberg, W.J. Storkus, and P. Kalinski. 2002. Complementary dendritic cell-activating function of CD8+ and CD4+ T cells: helper role of CD8+ T cells in the development of T helper type 1 responses. *The Journal of experimental medicine* 195:473-483.

Mangmool, S., and H. Kurose. 2011. G(i/o) protein-dependent and -independent actions of Pertussis Toxin (PTX). *Toxins* 3:884-899.

Markovic, D., and R.A. Challiss. 2009. Alternative splicing of G protein-coupled receptors: physiology and pathophysiology. *Cellular and molecular life sciences : CMLS* 66:3337-3352.

Marsango, S., M.C. Bonaccorsi di Patti, D. Barra, and R. Miele. 2011. Evidence that prokineticin receptor 2 exists as a dimer in vivo. *Cellular and molecular life sciences : CMLS* 68:2919-2929.

Martucci, C., S. Franchi, E. Giannini, H. Tian, P. Melchiorri, L. Negri, and P. Sacerdote. 2006. Bv8, the amphibian homologue of the mammalian prokineticins, induces a proinflammatory phenotype of mouse macrophages. *British journal of pharmacology* 147:225-234.

Masuda, Y., Y. Takatsu, Y. Terao, S. Kumano, Y. Ishibashi, M. Suenaga, M. Abe, S. Fukusumi, T. Watanabe, Y. Shintani, T. Yamada, S. Hinuma, N. Inatomi, T. Ohtaki, H. Onda, and M. Fujino. 2002. Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochemical and biophysical research communications* 293:396-402.

McMahon, S.B., W.B. Cafferty, and F. Marchand. 2005. Immune and glial cell factors as pain mediators and modulators. *Experimental neurology* 192:444-462.

McWhinney, C.D., M.W. Dudley, T.L. Bowlin, N.P. Peet, L. Schook, M. Bradshaw, M. De, D.R. Borcherding, and C.K. Edwards, 3rd. 1996. Activation of adenosine A3 receptors on macrophages inhibits tumor necrosis factor-alpha. *European journal of pharmacology* 310:209-216.

Melief, C.J. 2013. "License to kill" reflects joint action of CD4 and CD8 T cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19:4295-4296.

Merskey, H. 1979. Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy. *Pain* 6:249-252.

Merskey, H.B., N. 1994. Classification of chronic pain. 2nd ed. Seattle. *IASP Press*

Moalem, G., K. Xu, and L. Yu. 2004. T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. *Neuroscience* 129:767-777.

Mollay, C., C. Wechselberger, G. Mignogna, L. Negri, P. Melchiorri, D. Barra, and G. Kreil. 1999. Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. *European journal of pharmacology* 374:189-196.

Monassi, C.R., R. Bandler, and K.A. Keay. 2003. A subpopulation of rats show social and sleep-waking changes typical of chronic neuropathic pain following peripheral nerve injury. *The European journal of neuroscience* 17:1907-1920.

Monnier, C., C. Dode, L. Fabre, L. Teixeira, G. Labesse, J.P. Pin, J.P. Hardelin, and P. Rondard. 2009. PROKR2 missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Human molecular genetics* 18:75-81.

Monnier, J., and M. Samson. 2008. Cytokine properties of prokineticins. *The FEBS journal* 275:4014-4021.

Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology* 19:683-765.

Moran, M.M., M.A. McAlexander, T. Biro, and A. Szallasi. 2011. Transient receptor potential channels as therapeutic targets. *Nature reviews. Drug discovery* 10:601-620.

Morello, S., A. Petrella, M. Festa, A. Popolo, M. Monaco, E. Vuttariello, G. Chiappetta, L. Parente, and A. Pinto. 2008. Cl-IB-MECA inhibits human thyroid cancer cell proliferation independently of A3 adenosine receptor activation. *Cancer biology & therapy* 7:278-284.

National Research Council Committee, U. 2009. Recognition and Alleviation of Pain in Laboratory Animals. *National Academies Press (US)*

Negri, L., and R. Lattanzi. 2011. Bv8-prokineticins and their receptors: modulators of pain. *Current pharmaceutical biotechnology* 12:1720-1727.

Negri, L., and R. Lattanzi. 2012. Bv8/PK2 and prokineticin receptors: a druggable pronociceptive system. *Current opinion in pharmacology* 12:62-66.

Negri, L., R. Lattanzi, E. Giannini, M. Canestrelli, A. Nicotra, and P. Melchiorri. 2009. Bv8/Prokineticins and their Receptors A New Pronociceptive System. *International review of neurobiology* 85:145-157.

Negri, L., R. Lattanzi, E. Giannini, M. Colucci, F. Margheriti, P. Melchiorri, V. Vellani, H. Tian, M. De Felice, and F. Porreca. 2006. Impaired nociception and inflammatory pain sensation in mice lacking the prokineticin receptor PKR1: focus on interaction between PKR1 and the capsaicin receptor TRPV1 in pain behavior. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26:6716-6727.

Negri, L., R. Lattanzi, E. Giannini, M.A. Colucci, G. Mignogna, D. Barra, F. Grohovaz, F. Codazzi, A. Kaiser, G. Kreil, and P. Melchiorri. 2005. Biological activities of Bv8 analogues. *British journal of pharmacology* 146:625-632.

Negri, L., R. Lattanzi, E. Giannini, M. De Felice, A. Colucci, and P. Melchiorri. 2004. Bv8, the amphibian homologue of the mammalian prokineticins, modulates ingestive behaviour in rats. *British journal of pharmacology* 142:181-191.

Negri, L., R. Lattanzi, E. Giannini, and P. Melchiorri. 2007. Bv8/Prokineticin proteins and their receptors. *Life sciences* 81:1103-1116.

Negri, L., R. Lattanzi, E. Giannini, A. Metere, M. Colucci, D. Barra, G. Kreil, and P. Melchiorri. 2002. Nociceptive sensitization by the secretory protein Bv8. *British journal of pharmacology* 137:1147-1154.

Negri, L., and D. Maftei. 2018. Targeting the Prokineticin System to Control Chronic Pain and Inflammation. *Current medicinal chemistry* 25:3883-3894.

Neurath, M.F. 2014. Cytokines in inflammatory bowel disease. *Nature reviews. Immunology* 14:329-342.

Ngan, E.S., C.K. Shum, H.C. Poon, M.H. Sham, M.M. Garcia-Barcelo, V.C. Lui, and P.K. Tam. 2008. Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic

factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells. *Biochimica et biophysica acta* 1783:467-478.

Nicol, G.D., J.C. Lopshire, and C.M. Pafford. 1997. Tumor necrosis factor enhances the capsaicin sensitivity of rat sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 17:975-982.

Ochaion, A., S. Bar-Yehuda, S. Cohen, F. Barer, R. Patoka, H. Amital, T. Reitblat, A. Reitblat, J. Ophir, I. Konfino, Y. Chowers, S. Ben-Horin, and P. Fishman. 2009. The anti-inflammatory target A(3) adenosine receptor is over-expressed in rheumatoid arthritis, psoriasis and Crohn's disease. *Cellular immunology* 258:115-122.

Ochaion, A., S. Bar-Yehuda, S. Cohn, L. Del Valle, G. Perez-Liz, L. Madi, F. Barer, M. Farbstein, S. Fishman-Furman, T. Reitblat, A. Reitblat, H. Amital, Y. Levi, Y. Molad, R. Mader, M. Tishler, P. Langevitz, A. Zabutti, and P. Fishman. 2006. Methotrexate enhances the anti-inflammatory effect of CF101 via up-regulation of the A3 adenosine receptor expression. *Arthritis research & therapy* 8:R169.

Oh, S.B., P.B. Tran, S.E. Gillard, R.W. Hurley, D.L. Hammond, and R.J. Miller. 2001. Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21:5027-5035.

Ohsawa, K., T. Sanagi, Y. Nakamura, E. Suzuki, K. Inoue, and S. Kohsaka. 2012. Adenosine A3 receptor is involved in ADP-induced microglial process extension and migration. *Journal of neurochemistry* 121:217-227.

Ohta, A., and M. Sitkovsky. 2014. Extracellular adenosine-mediated modulation of regulatory T cells. *Frontiers in immunology* 5:304.

Oladosu, F.A., W. Maixner, and A.G. Nackley. 2015. Alternative Splicing of G Protein-Coupled Receptors: Relevance to Pain Management. *Mayo Clinic proceedings* 90:1135-1151.

Olah, M.E., C. Gallo-Rodriguez, K.A. Jacobson, and G.L. Stiles. 1994. 125I-4-aminobenzyl-5'-N-methylcarboxamidoadenosine, a high affinity radioligand for the rat A3 adenosine receptor. *Molecular pharmacology* 45:978-982.

Olah, M.E., and G.L. Stiles. 2000. The role of receptor structure in determining adenosine receptor activity. *Pharmacology & therapeutics* 85:55-75.

Ordovas-Montanes, J., S. Rakoff-Nahoum, S. Huang, L. Riol-Blanco, O. Barreiro, and U.H. von Andrian. 2015. The Regulation of Immunological Processes by Peripheral Neurons in Homeostasis and Disease. *Trends in immunology* 36:578-604.

Ossipov, M.H., K. Morimura, and F. Porreca. 2014. Descending pain modulation and chronification of pain. *Current opinion in supportive and palliative care* 8:143-151.

Paoletta, S., D.K. Tosh, A. Finley, E.T. Gizewski, S.M. Moss, Z.G. Gao, J.A. Auchampach, D. Salvemini, and K.A. Jacobson. 2013. Rational design of sulfonated A3 adenosine receptor-selective nucleosides as pharmacological tools to study chronic neuropathic pain. *Journal of medicinal chemistry* 56:5949-5963.

Parker, R., M. Liu, H.J. Eyre, N.G. Copeland, D.J. Gilbert, J. Crawford, G.R. Sutherland, N.A. Jenkins, and H. Herzog. 2000. Y-receptor-like genes GPR72 and GPR73: molecular cloning, genomic organisation and assignment to human chromosome 11q21.1 and 2p14 and mouse chromosome 9 and 6. *Biochimica et biophysica acta* 1491:369-375.

Pinho-Ribeiro, F.A., W.A. Verri, Jr., and I.M. Chiu. 2017. Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation. *Trends in immunology* 38:5-19.

Pizzo, P.A., and N.M. Clark. 2012. Alleviating suffering 101--pain relief in the United States. *The New England journal of medicine* 366:197-199.

Qiu, C.Y., Y.Q. Liu, F. Qiu, J. Wu, Q.Y. Zhou, and W.P. Hu. 2012. Prokineticin 2 potentiates acid-sensing ion channel activity in rat dorsal root ganglion neurons. *Journal of neuroinflammation* 9:108.

Qu, X., G. Zhuang, L. Yu, G. Meng, and N. Ferrara. 2012. Induction of Bv8 expression by granulocyte colony-stimulating factor in CD11b+Gr1+ cells: key role of Stat3 signaling. *The Journal of biological chemistry* 287:19574-19584.

Raghavan, S., A.D. Harvey, and S.R. Humble. 2011. New opioid side effects and implications for long-term therapy. *Trends in Anaesthesia and Critical Care* 1:18-21.

Ren, C., C.Y. Qiu, X. Gan, T.T. Liu, Z.W. Qu, Z. Rao, and W.P. Hu. 2015. Prokineticin 2 facilitates mechanical allodynia induced by alpha,beta-methylene ATP in rats. *European journal of pharmacology* 767:24-29.

Ren, K., and R. Dubner. 2010. Interactions between the immune and nervous systems in pain. *Nature medicine* 16:1267-1276.

Ru, F., L. Surdenikova, M. Brozmanova, and M. Kollarik. 2011. Adenosine-induced activation of esophageal nociceptors. *Am J Physiol Gastrointest Liver Physiol* 300:G485-493.

Ruiz-Ferrer, M., A. Torroglosa, R. Nunez-Torres, J.C. de Agustin, G. Antinolo, and S. Borrego. 2011. Expression of PROKR1 and PROKR2 in human enteric neural precursor cells and identification of sequence variants suggest a role in HSCR. *PloS one* 6:e23475.

Sah, D.W., M.H. Ossipo, and F. Porreca. 2003. Neurotrophic factors as novel therapeutics for neuropathic pain. *Nature reviews. Drug discovery* 2:460-472.

Salvatore, C.A., M.A. Jacobson, H.E. Taylor, J. Linden, and R.G. Johnson. 1993. Molecular cloning and characterization of the human A3 adenosine receptor. *Proceedings of the National Academy of Sciences of the United States of America* 90:10365-10369.

Scholz, J., and C.J. Woolf. 2007. The neuropathic pain triad: neurons, immune cells and glia. *Nature neuroscience* 10:1361-1368.

Sharma, S., B. Yang, X. Xi, J.C. Grotta, J. Aronowski, and S.I. Savitz. 2011. IL-10 directly protects cortical neurons by activating PI-3 kinase and STAT-3 pathways. *Brain research* 1373:189-194.

Shen, K.F., H.Q. Zhu, X.H. Wei, J. Wang, Y.Y. Li, R.P. Pang, and X.G. Liu. 2013. Interleukin-10 down-regulates voltage gated sodium channels in rat dorsal root ganglion neurons. *Experimental neurology* 247:466-475.

Sheth, S., R. Brito, D. Mukherjea, L.P. Rybak, and V. Ramkumar. 2014. Adenosine receptors: expression, function and regulation. *International journal of molecular sciences* 15:2024-2052.

Shojaei, F., X. Wu, X. Qu, M. Kowanetz, L. Yu, M. Tan, Y.G. Meng, and N. Ferrara. 2009. G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proceedings of the National Academy of Sciences of the United States of America* 106:6742-6747.

Shojaei, F., X. Wu, C. Zhong, L. Yu, X.H. Liang, J. Yao, D. Blanchard, C. Bais, F.V. Peale, N. van Bruggen, C. Ho, J. Ross, M. Tan, R.A. Carano, Y.G. Meng, and N. Ferrara. 2007. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450:825-831.

Silverman, M.H., V. Strand, D. Markovits, M. Nahir, T. Reitblat, Y. Molad, I. Rosner, M. Rozenbaum, R. Mader, M. Adawi, D. Caspi, M. Tishler, P. Langevitz, A. Rubinow, J. Friedman, L. Green, A. Tanay, A. Ochaion, S. Cohen, W.D. Kerns, I. Cohn, S. Fishman-Furman, M. Farbstein, S.B. Yehuda, and P. Fishman. 2008. Clinical evidence for utilization of the A3 adenosine receptor as a target to treat rheumatoid arthritis: data from a phase II clinical trial. *The Journal of rheumatology* 35:41-48.

Soga, T., S. Matsumoto, T. Oda, T. Saito, H. Hiyama, J. Takasaki, M. Kamohara, T. Ohishi, H. Matsushime, and K. Furuichi. 2002. Molecular cloning and characterization of prokineticin receptors. *Biochimica et biophysica acta* 1579:173-179.

Sowa, N.A., S.E. Street, P. Vihko, and M.J. Zylka. 2010. Prostatic acid phosphatase reduces thermal sensitivity and chronic pain sensitization by depleting phosphatidylinositol 4,5-bisphosphate. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30:10282-10293.

Sposini, S., G. Caltabiano, A.C. Hanyaloglu, and R. Miele. 2015. Identification of transmembrane domains that regulate spatial arrangements and activity of prokineticin receptor 2 dimers. *Molecular and cellular endocrinology* 399:362-372.

Stemmer, S.M., O. Benjaminov, G. Medalia, N.B. Ciuraru, M.H. Silverman, S. Bar-Yehuda, S. Fishman, Z. Harpaz, M. Farbstein, S. Cohen, R. Patoka, B. Singer, W.D. Kerns, and P. Fishman. 2013. CF102 for the treatment of hepatocellular carcinoma: a phase I/II, open-label, dose-escalation study. *The oncologist* 18:25-26.

Takaishi, K., J.H. Eisele, Jr., and E. Carstens. 1996. Behavioral and electrophysiological assessment of hyperalgesia and changes in dorsal horn responses following partial sciatic nerve ligation in rats. *Pain* 66:297-306.

Taliani, S., I. Pugliesi, M. Bellandi, C. La Motta, and F. Da Settimo. 2010. A3 receptor ligands: past, present and future trends. *Current topics in medicinal chemistry* 10:942-975.

Tappe-Theodor, A., and R. Kuner. 2014. Studying ongoing and spontaneous pain in rodents--challenges and opportunities. *The European journal of neuroscience* 39:1881-1890.

Terayama, R., M. Tabata, K. Maruhama, and S. Iida. 2018. A3 adenosine receptor agonist attenuates neuropathic pain by suppressing activation of microglia and convergence of nociceptive inputs in the spinal dorsal horn. *Experimental brain research*

Terayama, R., Y. Yamamoto, N. Kishimoto, K. Maruhama, M. Mizutani, S. Iida, and T. Sugimoto. 2015. Peripheral nerve injury activates convergent nociceptive input to dorsal horn neurons from neighboring intact nerve. *Experimental brain research* 233:1201-1212.

Thacker, M.A., A.K. Clark, T. Bishop, J. Grist, P.K. Yip, L.D. Moon, S.W. Thompson, F. Marchand, and S.B. McMahon. 2009. CCL2 is a key mediator of microglia activation in neuropathic pain states. *European journal of pain (London, England)* 13:263-272.

Thacker, M.A., A.K. Clark, F. Marchand, and S.B. McMahon. 2007. Pathophysiology of peripheral neuropathic pain: immune cells and molecules. *Anesthesia and analgesia* 105:838-847.

Todd, A.J. 2010. Neuronal circuitry for pain processing in the dorsal horn. *Nature reviews. Neuroscience* 11:823-836.

Torrance, N., J.A. Ferguson, E. Afolabi, M.I. Bennett, M.G. Serpell, K.M. Dunn, and B.H. Smith. 2013. Neuropathic pain in the community: more under-treated than refractory? *Pain* 154:690-699.

Tosh, D.K., S. Crane, Z. Chen, S. Paoletta, Z.G. Gao, E. Gizewski, J.A. Auchampach, D. Salvemini, and K.A. Jacobson. 2015a. Rigidified A3 Adenosine Receptor Agonists: 1-Deazaadenine Modification Maintains High in Vivo Efficacy. *ACS medicinal chemistry letters* 6:804-808.

Tosh, D.K., F. Deflorian, K. Phan, Z.G. Gao, T.C. Wan, E. Gizewski, J.A. Auchampach, and K.A. Jacobson. 2012. Structure-guided design of A(3) adenosine receptor-selective nucleosides: combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. *Journal of medicinal chemistry* 55:4847-4860.

Tosh, D.K., A. Finley, S. Paoletta, S.M. Moss, Z.G. Gao, E.T. Gizewski, J.A. Auchampach, D. Salvemini, and K.A. Jacobson. 2014. In vivo phenotypic screening for treating chronic neuropathic pain: modification of C2-arylethynyl group of conformationally constrained A3 adenosine receptor agonists. *Journal of medicinal chemistry* 57:9901-9914.

Tosh, D.K., J. Padia, D. Salvemini, and K.A. Jacobson. 2015b. Efficient, large-scale synthesis and preclinical studies of MRS5698, a highly selective A3 adenosine receptor agonist that protects against chronic neuropathic pain. *Purinergic signalling* 11:371-387.

Trautmann, A. 2009. Extracellular ATP in the immune system: more than just a "danger signal". *Science signaling* 2:pe6.

Treede, R.D. 2011. Entstehung der Schmerzchronifizierung. In: Baron R, Koppert W, Strumpf M, Willweber-Strumpf A, editors. , editors. Praktische Schmerztherapie. *Springer* 3-13.

Treede, R.D., W. Rief, A. Barke, Q. Aziz, M.I. Bennett, R. Benoliel, M. Cohen, S. Evers, N.B. Finnerup, M.B. First, M.A. Giamberardino, S. Kaasa, E. Kosek, P. Lavand'homme, M. Nicholas, S. Perrot, J. Scholz, S. Schug, B.H. Smith, P. Svensson, J.W. Vlaeyen, and S.J. Wang. 2015. A classification of chronic pain for ICD-11. *Pain* 156:1003-1007.

Tsuda, M., K. Inoue, and M.W. Salter. 2005. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends in neurosciences* 28:101-107.

Turovskaya, M.V., E.A. Turovsky, V.P. Zinchenko, S.G. Levin, and O.V. Godukhin. 2012. Interleukin-10 modulates [Ca2+]i response induced by repeated NMDA receptor activation with brief hypoxia through inhibition of InsP(3)-sensitive internal stores in hippocampal neurons. *Neuroscience letters* 516:151-155.

Ueda, H. 2006. Molecular mechanisms of neuropathic pain-phenotypic switch and initiation mechanisms. *Pharmacology & therapeutics* 109:57-77.

van Galen, P.J., A.H. van Bergen, C. Gallo-Rodriguez, N. Melman, M.E. Olah, I.J. AP, G.L. Stiles, and K.A. Jacobson. 1994. A binding site model and structure-activity relationships for the rat A3 adenosine receptor. *Molecular pharmacology* 45:1101-1111.

van Hecke, O., S.K. Austin, R.A. Khan, B.H. Smith, and N. Torrance. 2014. Neuropathic pain in the general population: a systematic review of epidemiological studies. *Pain* 155:654-662.

Vellani, V., M. Colucci, R. Lattanzi, E. Giannini, L. Negri, P. Melchiorri, and P.A. McNaughton. 2006. Sensitization of transient receptor potential vanilloid 1 by the prokineticin receptor

agonist Bv8. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26:5109-5116.

Von Frey, M. 1896. On the Use of Stimulus Hairs. 1896:71–131.

von Hehn, C.A., R. Baron, and C.J. Woolf. 2012. Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron* 73:638-652.

Vos, B.P., A.M. Strassman, and R.J. Maciewicz. 1994. Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 14:2708-2723.

Wahlman, C., T.M. Doyle, J.W. Little, L. Luongo, K. Janes, Z. Chen, E. Esposito, D.K. Tosh, S. Cuzzocrea, K.A. Jacobson, and D. Salvemini. 2018. Chemotherapy-induced pain is promoted by enhanced spinal adenosine kinase levels through astrocyte-dependent mechanisms. *Pain* 159:1025-1034.

Walsh, J.T., S. Hendrix, F. Boato, I. Smirnov, J. Zheng, J.R. Lukens, S. Gadani, D. Hechler, G. Golz, K. Rosenberger, T. Kammertons, J. Vogt, C. Vogelaar, V. Siffrin, A. Radjavi, A. Fernandez-Castaneda, A. Gaultier, R. Gold, T.D. Kanneganti, R. Nitsch, F. Zipp, and J. Kipnis. 2015. MHCII-independent CD4+ T cells protect injured CNS neurons via IL-4. *The Journal of clinical investigation* 125:699-714.

Wan, T.C., Z.D. Ge, A. Tampo, Y. Mio, M.W. Bienengraeber, W.R. Tracey, G.J. Gross, W.M. Kwok, and J.A. Auchampach. 2008. The A3 adenosine receptor agonist CP-532,903 [N6-(2,5-dichlorobenzyl)-3'-aminoadenosine-5'-N-methylcarboxamide] protects against myocardial ischemia/reperfusion injury via the sarcolemmal ATP-sensitive potassium channel. *The Journal of pharmacology and experimental therapeutics* 324:234-243.

Wang, Y., J. Liu, B.O. Huang, Y.M. Xu, J. Li, L.F. Huang, J. Lin, J. Zhang, Q.H. Min, W.M. Yang, and X.Z. Wang. 2015. Mechanism of alternative splicing and its regulation. *Biomedical reports* 3:152-158.

Watson, R.P., E. Lilley, M. Panesar, G. Bhalay, S. Langridge, S.S. Tian, C. McClenaghan, A. Ropenga, F. Zeng, and M.S. Nash. 2012. Increased prokineticin 2 expression in gut inflammation: role in visceral pain and intestinal ion transport. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society* 24:65-75, e12.

Waxman, S.G., and G.W. Zamponi. 2014. Regulating excitability of peripheral afferents: emerging ion channel targets. *Nature neuroscience* 17:153-163.

Wechselberger, C., R. Puglisi, E. Engel, G. Lepperdinger, C. Boitani, and G. Kreil. 1999. The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes. *FEBS letters* 462:177-181.

Willemen, H.L., N. Eijkelkamp, A. Garza Carbajal, H. Wang, M. Mack, J. Zijlstra, C.J. Heijnen, and A. Kavelaars. 2014. Monocytes/Macrophages control resolution of transient inflammatory pain. *The journal of pain : official journal of the American Pain Society* 15:496-506.

Xanthos, D.N., and J. Sandkuhler. 2014. Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. *Nature reviews. Neuroscience* 15:43-53.

Xiong, Y.C., X.M. Li, X.J. Wang, Y.Q. Liu, F. Qiu, D. Wu, Y.B. Gan, B.H. Wang, and W.P. Hu. 2010. Prokineticin 2 suppresses GABA-activated current in rat primary sensory neurons. *Neuropharmacology* 59:589-594.

Yaar, R., E.D. Lamperti, P.A. Toselli, and K. Ravid. 2002. Activity of the A3 adenosine receptor gene promoter in transgenic mice: characterization of previously unidentified sites of expression. *FEBS letters* 532:267-272.

Yamamoto, Y., R. Terayama, N. Kishimoto, K. Maruhama, M. Mizutani, S. Iida, and T. Sugimoto. 2015. Activated microglia contribute to convergent nociceptive inputs to spinal dorsal horn neurons and the development of neuropathic pain. *Neurochemical research* 40:1000-1012.

Yan, H., E. Zhang, C. Feng, and X. Zhao. 2016. Role of A3 adenosine receptor in diabetic neuropathy. *Journal of neuroscience research* 94:936-946.

Yoon, M.H., H.B. Bae, and J.I. Choi. 2005. Antinociception of intrathecal adenosine receptor subtype agonists in rat formalin test. *Anesthesia and analgesia* 101:1417-1421.

Zhang, F.F., N. Morioka, S. Harano, Y. Nakamura, K. Liu, M. Nishibori, K. Hisaoka-Nakashima, and Y. Nakata. 2016. Perineural expression of high-mobility group box-1 contributes to long-lasting mechanical hypersensitivity via matrix metalloprotease-9 up-regulation in mice with painful peripheral neuropathy. *Journal of neurochemistry* 136:837-850.

Zheng, J., R. Wang, E. Zambraski, D. Wu, K.A. Jacobson, and B.T. Liang. 2007. Protective roles of adenosine A1, A2A, and A3 receptors in skeletal muscle ischemia and reperfusion injury. *American journal of physiology. Heart and circulatory physiology* 293:H3685-3691.

Zhou, Z., X. Peng, R. Insolera, D.J. Fink, and M. Mata. 2009a. IL-10 promotes neuronal survival following spinal cord injury. *Experimental neurology* 220:183-190.

Zhou, Z., X. Peng, R. Insolera, D.J. Fink, and M. Mata. 2009b. Interleukin-10 provides direct trophic support to neurons. *Journal of neurochemistry* 110:1617-1627.

Zylka, M.J. 2011. Pain-relieving prospects for adenosine receptors and ectonucleotidases. *Trends in molecular medicine* 17:188-196.

7. ACKNOWLEDGEMENTS

Un primo doveroso ringraziamento va alla Prof.ssa Lucia Negri e alla Prof.ssa Roberta Lattanzi, per avermi accolto nel loro laboratorio dandomi la possibilità di svolgere questo lavoro di tesi. Grazie per aver scommesso su questa "piccolina" venuta da fuori e per avermi coinvolta durante questi 3 anni in molti interessanti progetti, contribuendo così alla mia formazione scientifica. Ma soprattutto grazie per avermi dato un'opportunità che non capita a tutti: quella di trascorrere un anno di ricerca all'estero.

Un particolare ringraziamento va alla Prof.ssa Daniela Salvemini, per avermi fatto da mentore durante il mio secondo anno di dottorato. Grazie per tutti gli insegnamenti, per il tempo che mi ha dedicato e la fiducia che ha riposto nel mio lavoro. Alla Saint Louis University ho vissuto un'esperienza che più di ogni altra finora mi ha fatto crescere professionalmente e personalmente. Vorrei ringraziare anche Tim, Caron e tutti i ragazzi del laboratorio, perché ognuno con il proprio carattere e con il proprio bagaglio di conoscenze ha contribuito a rendere speciale questa esperienza.

Vorrei ringraziare la mia post doc preferita, Daniela, che tra noi due è la "vera" bilancia, con la sua calma e la sua passione per questo lavoro ha saputo guidarmi, insegnandomi davvero tantissimo in questi anni, sostenendomi quando pensavo non ce l'avrei fatta. Un ringraziamento particolare va anche a Veronica ed Ilaria per la loro pazienza ed il loro entusiasmo e ad Anamaria ed Alessandro per avermi fatto capire quanto sia bello imparare insegnando agli altri.

Credo sia impossibile spiegare a parole l'affetto che provo per Luisa, Riccardo, Mirella e Fabrizio, che negli ultimi 15 mesi del mio percorso di dottorato sono stati veramente la mia forza, un regalo bellissimo ed inaspettato. Non posso non ringraziare anche Martina, che ogni giorno, insieme agli altri, ha saputo strappare un sorriso a questa neurobiocosa.

Ai miei strepitosi genitori e ai miei nonni che ancora una volta hanno creduto in questa piccola biologa, grazie per il vostro dolce e instancabile sostegno e per aver condiviso con me tutte le forti emozioni che si sono succedute in questi anni. A zio Giorgio, zia Orietta e Flavia, grazie perché siete la prova che i legami sinceri resistono al tempo e alla distanza.

Un ringraziamento speciale ad Antonello, che meriterebbe davvero questo titolo *ad honorem* dal momento che, più di ogni altro, ha vissuto in prima persona insieme alla sottoscritta questo percorso.

Grazie per aver creduto in me, per non avermi mai frenata, ma sempre incoraggiata, per aver contribuito a costruire la fiducia che oggi ho in me stessa, mi sento immensamente fortunata ad averti al mio fianco. Vorrei ringraziare anche il mio fratellino Dario e tutta la famiglia De Angelis, che mi fa sentire sempre a casa.

Al mio cous, che è la mia roccia ed un pezzettino del mio cuore, grazie per essere stato sempre presente, è bellissimo crescere insieme.

Vorrei infine ringraziare tutti gli amici perché questo traguardo lo devo anche a loro, ai miei Aleffi che da anni sono il mio rifugio sicuro, con tanto affetto e tanta birra abbiamo superato anche la distanza; alle ragazze “di Costano” per essere la mia isola felice quando ho bisogno di scappare dalla frenesia, alla mia migliore amica Elisa, che con uno sguardo ha sempre saputo leggere dentro di me.

Portare a termine questo impegnativo percorso ha avuto un forte impatto sulla mia maturazione scientifica e sulla mia personalità. Considerando tutte le esperienze che ho vissuto e tutte le persone che ho avuto la possibilità di incontrare, posso dire senza ombra di dubbio che tornando indietro rifarei esattamente le stesse scelte.