PARTICIPATION OF THE ENDOGENOUS OPIOID AND CANNABINOID SYSTEMS IN NEUROPATHIC PAIN

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NeuroPhar

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Abbreviations

2-AG	2-arachidonoylglycerol
A2A	Adenosine type 2A receptor
AM404	*N*-(4-hydroxyphenyl)-arachidonamide
ATP	Adenosine triphosphate
BNDF	Brain derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CBD	Cannabidiol
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CPRS	Complex regional pain syndromes
DOR	Delta opioid receptor
DRG	Dorsal root ganglion
DSE	Depolarization-induced excitation
DSI	Depolarization-induced inhibition
FAAH	Fatty acid amide hydrolase
FABP	Faty Acid Binding Protein
GABA	Gamma aminobutiric acid
GPR55	G-protein receptor 55
IASP	International Association for the Study of Pain
IFN-γ	Interferon-gamma
IL-1β	Interleukin 1β
IL-6	Interleukin 6
INF-α	Interferon-alfa
iNOS	Inducible nitric oxide syntase
KCC	Potassium-chloride exporter
KO	Knockout

KOR	Kappa opioid receptor
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
MOR	Mu opioid receptor
MRI	Magnetic resonance imaging
NADA	N-arachidonoyldopamine
Nav	Voltage-gated sodium channels
NGF	Nerve grow factor
NK1	Neurokinin receptor 1
NMDA	N-methyl- D-aspartate
NOP	Nociceptin / orphanin receptor
ORL1	Orphanin-receptor like 1
P2X4	Purinergic ionotropic 4 receptor
PAG	Periaqueductal gray matter
PDYN	Prodynorphin
PENK	Proenkephalin
PET	Positron emission tomography
PKA	Protein kinase A
POMC	Proopiomelanocortin,
PSNL	Partial sciatic nerve ligation model
RVM	Rostroventromedial medulla
SNL	Spinal nerve ligation model
TNF	Tumour necrosis factor
TrkB	Tyrosine kinase B receptors
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid receptor 1
Δ 9-THC	Delta-9-tetrahydrocannabinol

Abstract

This work is focused in the study of the pathophysiology of neuropathic pain, particularly in the role of the endogenous opioid and cannabinoid systems. Neuropathic pain is a chronic illness with a high prevalence in the population and is characterized by the presence of spontaneous pain and abnormal stimulus-evoked pain responses, among other symptoms. It is a clinical pain manifestation that has shown to be poorly treated with the available pharmacological treatment. Even with the existence of many therapeutic approaches, there is not an adequate effective treatment for palliating all symptoms of neuropathic pain. This situation leads us to study the specific involvement of the endogenous opioid and cannabinoid systems in the pathophysiology of the development and maintenance of neuropathic pain. In the present study, we have evaluated the role of delta opioid receptor (DOR) in the central nervous system (CNS) and peripheral nociceptive neurons, as well as the participation of cannabinoid receptor type 2 (CB_2) in the activated microglia at the spinal cord. The results show that DOR and CB_2 may be pharmacological targets for the development of new drugs with analgesic activity, but devoid of the psychotropic side effects of traditional opioids and cannabinoid agonists.

Resum

Aquest treball es centra en l'estudi de la fisiopatologia del dolor neuropàtic, en particular en el paper dels sistemes endògens opioide i cannabinoide. El dolor neuropàtic és una malaltia crònica amb una alta prevalença en la població i es caracteritza per la presència de dolor espontani i percepció anormal del dolor, entre d'altres símptomes. És una manifestació clínica del dolor que ha demostrat ser mal tractada amb el tractament farmacològic disponible. Malgrat l'existència de molts enfocs terapèutics, no hi ha un tractament eficaç adequat per pal·liar els símptomes del dolor neuropàtic. Aquesta situació ens porta a estudiar la participació específica dels sistemes endògens opioide i cannabinoide en la fisiopatologia del desenvolupament i manteniment del dolor neuropàtic. En el present estudi, hem avaluat el paper del receptor opioide delta (DOR) en el sistema nerviós central (SNC) i perifèric en neurones nociceptives, així com la participació dels receptors cannabinoides tipus 2 (CB_2) a la microglia activada a la medul·la espinal. Els resultats mostren que DOR i CB_2 poden ser dianes farmacològiques per al desenvolupament de nous fàrmacs amb activitat analgèsica, però amb menys efectes psicotròpics secundaris dels opioides tradicionals i els agonistes cannabinoides.

INTRODUCTION

1. Pain: General considerations

a. Historical aspects

Pain is something inherent in the human condition. It was not until the Greek civilization that a scientific approach to pain started to arise. Two prevalent theories were present among the Greek scientists. The first was instaured by Plato, among others, and defended the brain as a centre of sensations and thinking, and explained pain as a sensation. The second, defended by Aristotle and Empedocles, theorised that the noxious stimuli travel from the skin to the heart through the blood, and the pain would be an emotion more than a sensation. The Roman physician Galen elaborated a complex theory of the sensations defending the brain as a receptor and a modulator of sensations, including pain. However, the Aristotelian theory of pain as an emotion prevailed until the XVI century, when Descartes retook the Galen's concept and confronted it with the Aristotle's one. Descartes theorised that the sensations travel through the nerves to the brain, were they were made conscious in the pineal gland (Bosch and Baños 2009).

The first modern theories about pain appeared in the XIX century. In 1840 Müller announced the theory of specific energies, and postulates that the nerves transmitted the external and internal information to the brain, and every sensory nerve gave a specific sensation when stimulated. Later it appeared the theory of nerve specificity of Von Frey, which postulated the specificity of the nerve pathways from the periphery to the central nervous system (CNS). Therefore, the pain, the cold, the hot and the tactile pathway, among others, might be distinguished. In conflict with that theory, in 1894 it appeared the summation theory of Goldsheider, what

postulates the inexistence of the specific pain pathways. By contrast, he maintained that pain is the consequence of the summation of impulses from other sensory pathways (Serra 2006).

In the XX century science was advancing in giant steps with important discoverings that contributed to the current knowledge about pain. One of the most important theories derived from then was the gate control theory (Melzack and Wall 1965). This theory maintains the existence of the nociceptive fibers that convey pain sensations, but these fibers converge in the spinal cord with afferent nerves, which transmit tactile sensations. Both stimulate the same wide dynamic range neurons. In consequence, the sustained activation secondary to nociceptive neuron injury can sensitize wide dynamic range neurons, and then the touch sensations can be felt as painful. This theory introduced the concept of spinal and supraespinal modulation of the stimulus, as well as the bidirectional interactions of sensations. Later on, Melzack proposed the neuromatrix theory, which defends the existence of a network of neuronal circuits prepared to receive sensorial nociceptive inputs, located in the limbic system and connected to multiple brain areas. Those neuronal circuits generate the emotional component of pain (Melzack 1971). Finally, the two old theories of pain converged in the present neuroanatomy and physiology of pain. Pain is not only a sensation, as the emotional component has an important role in the final perception of pain.

b. Definition

The definition proposed for the International Association for the Study of Pain (IASP) is currently the most well accepted. It describes pain as “an unpleasant sensory and emotional experience associated with actual or

potential tissue damage, or described in terms of such damage" (Merskey and Bogduk 1994). Therefore, pain cannot be only defined as a nociceptive experience because it is always a subjective experience, as well. Indeed, pain integrates different sensations, behaviours and thoughts that finally construct the symptom of pain. From the IASP definition, we can consider the existence of two principal components in pain (Bosch and Baños 2009):

- Nociceptive or sensorial: secondary to the transmission of painful stimuli from nerves to the brain cortex.
- Affective or reactive: the suffering associated to pain that can vary greatly depending of the cause, the moment and the experience of the patient, among many other factors. Many psychological factors can also modify the perception of the painful experience.

c. Classification based on pathophysiological mechanisms

Pain has been classified in many ways considering, among others, the duration (acute, chronic), the intensity (mild, moderate, severe), the localization (cervical, spinal, pelvic, leg, arm, shoulder) or the association to disease (rheumatism, cancer, neuropathic). For the purpose of the aims of this thesis, we prefer to consider the classical classification of Cerveró and Laird based in pathophysiological mechanisms (Cervero 1991). These authors identified three types of pain: nociceptive, inflammatory and neuropathic.

Nociceptive pain refers to that caused by brief and noxious stimuli which are followed by transient stimulation of nociceptive pathways, without

significant tissue injury. By contrast, inflammatory pain is the consequence of tissue damage due to the action of trauma events (surgery), physical (sun, heat) or chemical (acids, alkalis) agents. The injury that follows triggers mechanisms of repair that produces pain. It should be remembered that Celsus, the famous Roman physician, described pain as one of the four cardinal signs of inflammation in the I century a.C. In this process of repair, many molecules are released and act on the nociceptive fibers to produce pain (for instance, bradikinin) or to sensitize them by means of reducing their threshold activation. In this type of pain, hyperalgesia and allodynia are present as a consequence of peripheral or central sensitization. Hyperalgesia means that painful stimuli of low intensity are perceived as they were of high intensity. In the case of allodynia, non-painful stimuli (i.e. touch or temperature) trigger the sensation of pain. Once the process of healing has finished pain usually disappears, although in some cases it may persist leading to chronic pain.

The third type is neuropathic pain, which is consequence of neural damage, both at peripheral nervous system, such as neuropathies, or at the CNS, such as spinal injury or thalamic stroke. As a consequence, hyperalgesia and severe allodynia appear, and the relationship between stimulus and painful response is almost completely lost. The mechanisms that participate in each type of pain have been recently reviewed by Cerveró (Cervero 2009).

Some authors have added a fourth type of pain, named functional pain (Woolf 2004), that describes the situations when an injury occurs in a different place where the patient refers the presence of pain. Fibromyalgia, irritable bowel syndrome and some types of back pain may be included under this category. It seems that the most probable mechanism might be

an alteration in the processing of peripheral stimuli at the CNS, which may be linked to inhibitory systems.

d. Pain: a trip from periphery to the cerebral cortex

The nociceptors are located in the terminals of afferent neurons responsible for the pain stimuli detection and subsequent transmission. Such as all primary afferent neurons, neurons that express nociceptors, have a cellular body located in the dorsal root ganglion (DRG) and two prolongations (Figure 1). The central prolongation ends into the dorsal horn of the spinal cord whereas the peripheral prolongation ends in the peripheral organs and constitutes the sensory fiber. Taking into account the myelinization, the diameter and the conduction speed, the cutaneous sensory fibers are classified in three categories: Aβ fibers (large myelinated afferents), Aδ fibers (small myelinated afferents) and C fibers (small unmyelinated afferents). In physiological conditions, all of these fibers can transmit innocuous information, whereas only Aδ and C fibers transmit nociceptive information. When a nociceptive stimulus acts on the skin, the nociceptors in Aδ fibers are responsible for the transmission of the immediate acute pain. This is followed by a more diffused pain transmitted by the activation of nociceptors located on C fibers and characterised by a slower conduction speed. During chronic pain, nociceptor sensitization is caused by inflammatory mediators, such as amines, prostaglandins, leukotrienes and bradykinins that are released after injury, which results in a decrease of the threshold of nociceptive stimuli and in an increase of the response to suprathreshold stimuli (Banos et al. 2003). The responces of nociceptors are not the same in every type of pain. In nociceptive pain, the stimulus is short in duration, the activation of nociceptors conveys the signal to the CNS and subjects

experiment pain as an acute event that ends quickly. No tissue injury or either persistent activation ensues and all process finishes in seconds or minutes.

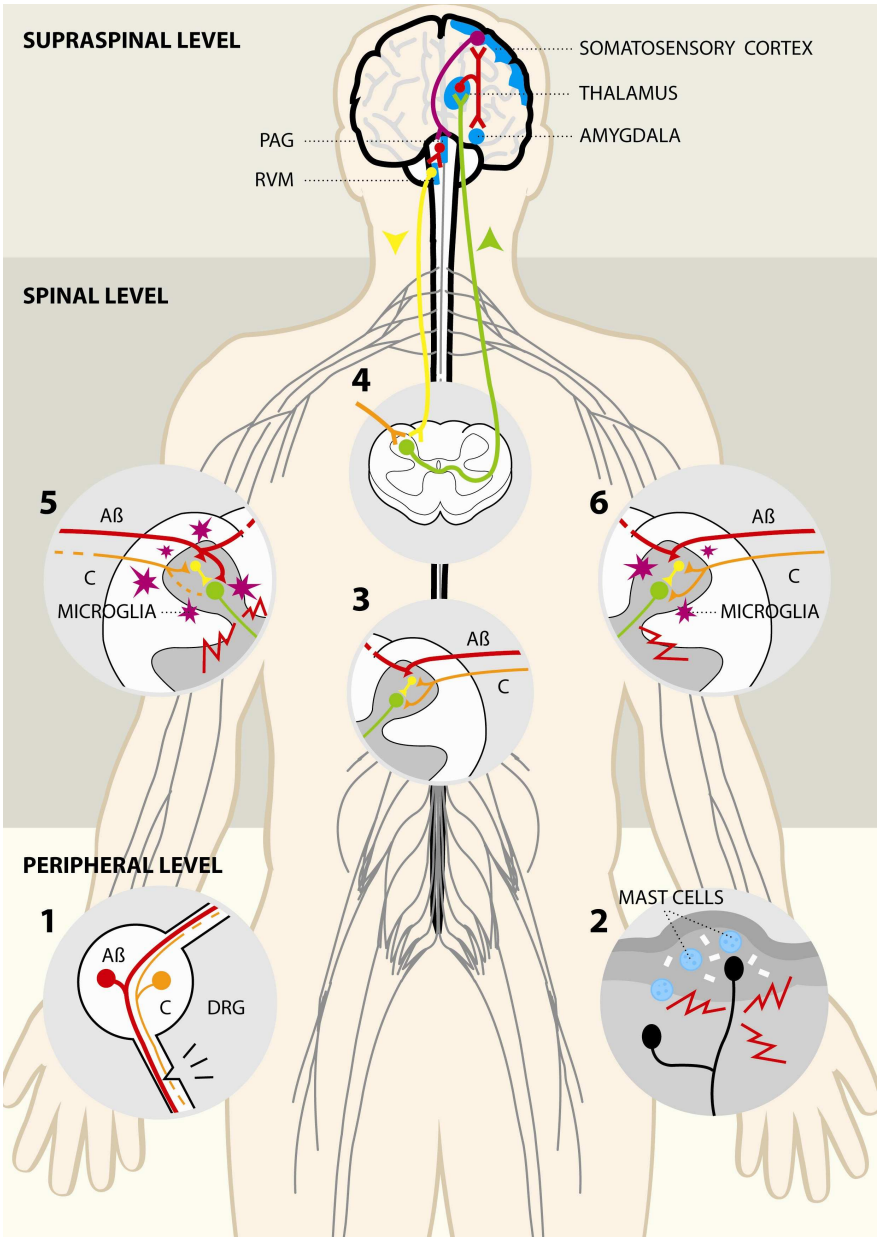

Figure 1. Nociceptive pathways and pathophysiology of neuropathic and inflammatory pain. Peripheral level: the painful stimulus is caused by the activation of nociceptive receptors in the nerve endings. 1: neuropathic pain in

nerve injury causes a adaptative changes of C fibers causing the painful stimulus, 2, while in inflammatory pain, mast cells infiltrate the tissue releasing proinflammatory factors that produce sensitization of nociceptive receptors. Spinal level, 3 and 4: the pain stimulus is transmitted to the spinal cord and hence will be conducted by the fibers that transmit pain signals to the brain, 5 and 6: an activation of microglia occurs in the spinal cord during both neuropathic pain and inflammatory pain that releases inflammatory cytokines orchestrating a neuroinflammatory process that produces neural sensitization, 5: In addition, in neuropathic pain, this neuroinflammation appears responsible for the abnormal growth of Aß fibers, which contributed to the phenomena of allodynia and hyperalgesia. Supraspinal level: the pain stimulus is transmitted from the spinal cord to the thalamus and hence to the amygdala, and somatosensory cortex. Here, we produce the subjective feeling of pain. These stimuli activate the periaqueductal grey matter (PAG) and the rostroventromedial medulla (RVM), which are the most important centers of descending inhibitory circuit. The final event is a reduction of painful stimuli wich reach the brain. (Adapted from Nadal and Baños 2009)

Inflammatory pain involves an important immune component. Mast cells and lymphocytes infiltrate into the tissue releasing pro-inflammatory molecules that sensitize the nociceptive receptors (Figure 1). In both types of pain, the activation of nociceptors conveys the signal to the CNS through dorsal root ganglion (DRG). The organism, in an attempt to repair the damage, activates the growth of Aβ fibers to innervate the neurons receiving the lost signal from the C fibers. The consequence is a change in the characteristics of response to tactile stimuli that will be perceived as painful (mechanical allodynia). This situation triggers the tactile stimulation of the Aβ fibers, which now is sent to the neurons of the spinal transmission circuit painful upward, producing the phenomena of allodynia and hyperalgesia (Woolf 1993) (Figure 1). In the spinal cord, the incoming stimulus releases neurotransmitters, such as N-methyl-D-aspartate (NMDA) or substance P, which activates second neurons. If the stimulus has enough intensity, this neuron transmits the pain sensation to the ascending pathways until supraspinal areas through specific pathways, such as spinothalamic, spinomesencephalic and spinoreticular tracts. In the spinal cord, the activation of neurons may be submitted to inhibition

by local interneurons and descending pathways. Only if inhibition is not strong enough, the painful stimulus is transmitted to ascending pathways. Besides the participation of neurotransmitters, an activation of microglia occurs in the spinal cord in some models of postoperative, neuropathic and inflammatory pain (Watkins et al. 2001; Tsuda et al. 2005). Microglial cells induce the release of inflammatory cytokines, which trigger inflammatory processes and neural sensitization (Figure 1). This neuroinflammation contributes to the growth of the fibers Aβ in neuropathic pain.

Painful stimuli are transmitted from the spinal cord to supraspinal structures, with an outstanding participation of the thalamus. Finally, this area connects with many brain structures, such as amygdala and somatic sensory cortex, which makes a complex network with almost all the brain structures. Many of these connections are responsible of the emotional component of pain (Figure 1). The affective dimension of pain includes feelings of unpleasantness and emotions. Spinal pathways to limbic structures and medial thalamic nuclei provide direct inputs to brain areas involved in emotional control. Another sources participating in this emotional component are the projections from spinal pathways to somatosensory thalamic and cortical areas, and then through a corticolimbic pathway. The latter integrates nociceptive input with contextual information and memory to provide cognitive mediation of pain affect. Both direct and corticolimbic pathways converge on the same anterior cingulate cortical and subcortical structures whose function may be to establish emotional balance and response priorities (Price 2000).

The CNS closely modulates the transmision of pain stimuli through the descending pathways, which may stimulate or, mostly, inhibit pain perception. These inhibitory mechanisms came from midbrain

periaqueductal gray matter (PAG) and rostroventromedial medulla (RVM) sending projections to the spinal cord, where they inhibit the entry of pain stimuli from peripheral nerves. These mechanisms may explain the action of some drugs (e.g. antidepressants) and the modulation of supraespinal centers on pain transmission.

2. Neuropathic pain

a. Definition and classification

IASP defines neuropathic pain as pain initiated or caused by a primary lesion or dysfunction in the nervous system. It is characterized from the clinical point of view by an array of abnormal responses to painful stimuli (hyperalgesia, allodynia) and spontaneous pain (Bridges et al. 2001). These abnormal pain sensations are associated with complex physiological changes in the peripheral nervous system and/or the CNS. They include spontaneous neuron discharging, alteration of ion channel expression, sprouting of primary afferent neurons, peripheral and central sensitisation, spinal reorganization and changes in inhibitory pain descending pathways (Woolf and Mannion 1999).

The classification of neuropathic pain is a complex matter. A traditional approach classifies neuropathic pain according to the aetiology, as well as the presumed location of the nerve injury (peripheral or central). The aetiology-based classification of neuropathic pain can be summarized as follows (Baron 2006):

- *Focal or multifocal lesions of the* peripheral nervous system*:* entrapment syndromes, phantom limb pain, stump pain, post-traumatic neuralgia, postherpetic neuralgia, diabetic mononeuropathy, ischemic neuropathy and polyarteritis nodosa.
- *Generalized lesions of the* peripheral nervous system *(polyneuropathies):* diabetes mellitus, cancer-associated neuropathy, amyloid plasmocytoma, HIV neuropathy, hypothyroidism, hereditary sensory neuropathies, vitamin B

deficiency, toxic neuropathies (alcohol, arsenic, thallium, chloramphenicol, metronidazole, nitrofurantoin, isoniazid, vinca alkaloids and taxol, among others), Fabry's disease and Bannwarth's syndrome (neuroborreliosis).

- *Lesions of the CNS:* spinal cord injury, brain infarction (especially in the thalamus and brainstem), spinal infarction, syringomyelia and multiple sclerosis.
- *Complex neuropathic disorders:* complex regional pain syndromes type I and II (previously known as reflex sympathetic dystrophy and causalgia).

b. Clinical characteristics: signs and symptoms

As stated before, neuropathic pain is characterized by the existence of spontaneous pain and abnormal stimulus-evoked pain (mainly hyperalgesia and allodynia, but also paresthesia and dysesthesia). When a stimulus that usually causes mild pain is perceived by patient as producing severe pain, this situation is called hyperalgesia. Depending on the nature of the stimulus, the resultant condition is known as heat, cold or mechanical hyperalgesia. However, in some cases painless stimuli (such as the rubbing of clothing) are felt as painful, and this situation is known as allodynia, that may be very distressing for some patients. Besides, hyperalgesia and allodynia, there are other evoked sensory phenomena, such as paresthesia (abnormal sensation, different from pain, whether spontaneous or evoked, not unpleasant) or dysesthesia (abnormal and unpleasant sensation, different from pain whether spontaneous or evoked, unpleasant) (Banos et al. 2003). Another characteristic in some cases of neuropathic pain, like entrapment neuropathies, is the appearance of Tinel's sign, which is very useful to determine the anatomical level of the

lesion of a nerve injury. This is a tingling sensation in the distal end of a limb when percussion is made over the site of a divided nerve.

c. Pathophysiology

Most of the current hypothesis to explain the pathophysiology and mechanisms underlying neuropathic pain originated from experimental work in animal models. These studies have delineated a series of partially independent peripheral and central mechanisms to explain the development and manifestations of neuropathic pain (Baron 2006).

i. Peripheral mechanisms

After a peripheral nerve lesion, the nociceptors become abnormally sensitive and develop a spontaneous activity. These pathological changes are underpinned by molecular and cellular modifications at the primary afferent nociceptor that are triggered by the nerve injury (Baron 2006). The consequences are ectopic (abnormal) and spontaneous discharges, abnormal nerve conduction, alterations of ionic channel expression, collateral spouting of primary afferent neurons, sprouting of sympathetic neurons and nociceptor sensitisation (Banos et al. 2003)

The ectopic and spontaneous discharges are expressed as a large increase in spontaneous firing in the afferent neurons linked to the injury site (Wall and Gutnick 1974; Wall and Devor 1983). At least two subpopulations of primary afferents develop ectopic activity in the presence of nerve injury: injured afferent neurons and their uninjured neighbours. Thus, both populations of afferents are hypothetically capable of initiating, as well as maintaining, the behavioural changes observed in the presence of nerve

injury (Gold 2000a; Gold 2000b). These abnormal discharges can be spontaneous and due to instability of the membrane potential or caused by undetectable stimuli.

Ectopic and spontaneous activity following nerve injury is matched by increased expression of messenger RNA for voltage-gated sodium channels (Nav) in the primary afferent neurons. Clustering of sodium channels at sites of ectopic impulse generation might be responsible for the lowering of the action-potential threshold and consequent hyperactivity (Lai et al. 2003). The genes that encode the Nav are expressed selectively in nociceptive primary afferent neurons (Wood 2004). After peripheral nerve damage, Nav clusters accumulate not only at the site of the nerve lesion, but also within the intact DRG. Within the DRG, an alternation between a phasically activating voltage-dependent tetrodotoxin sensitive sodium conductance and a passive voltage-independent potassium leak generates characteristic membrane potential oscillations (Amir et al. 2002).

Damage to peripheral nerves also induces up-regulation of several receptors, some of which are only marginally expressed under physiological conditions at the membrane of primary afferents. Thus, partial nerve injury and streptozotocin-induced diabetes produce a down-regulation of vanilloid receptors type 1 (TRPV1) on many damaged afferent neurons and novel expression of TRPV1 on uninjured C fibers and A fibers (myelinated Aβ and Aδ) (Hudson et al. 2001; Hong and Wiley 2006). Recent studies also reveal an up-regulation of TRPV1 in medium and large injured DRG cells (Ma et al. 2005). TRPV1 are located predominantly on nociceptive afferent fibers and transmit noxious heat (>43°C) (Caterina et al. 2000). The observation that TRPV1-deficient mice do not develop heat hyperalgesia after tissue inflammation (Caterina

et al. 2000; Davis et al. 2000) supports the idea that these changes might contribute to the development of peripheral sensitization and the associated heat hyperalgesia (Baron 2000). TRPV1 does not seem to be the only transduction mechanism for thermal sensitization after nerve injury since wild type and TRPV1-null mice exhibited comparable persistent enhancement of mechanical and thermal nociception after partial sciatic nerve ligation (Caterina et al. 2000).

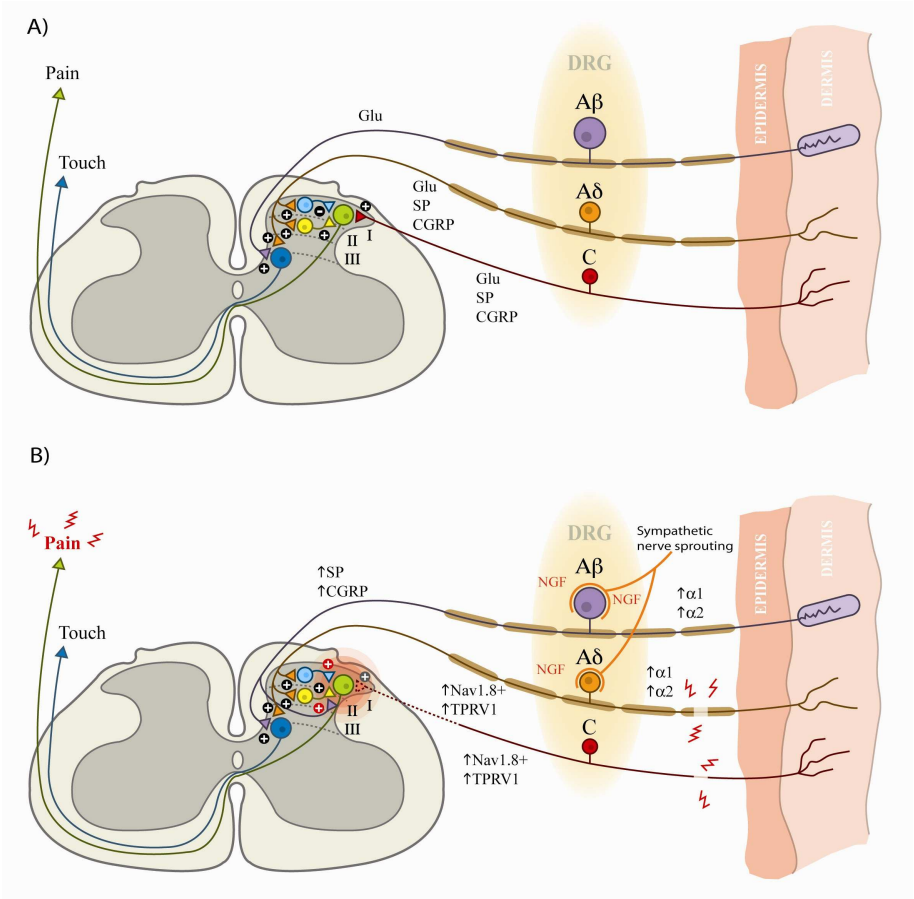

Figure 2. Nociceptive pathways at peripheral and spinal level. A) Normal transition of diffuse nociceptive stimulus is done by C fibers that projects to the lamina I neurons (green neuron) which transmit painful stimulus. The fast and localized painful stimuli are transmitted by the Aδ fibers that connect to the lamina II glutamatergic and substance P excitatory interneuron (yellow neuron) that excites the nociceptive neuron (green). Aδ fibers can innervate to the GABAergic and enkephalinergic interneurons (blue neuron) modulating the

nociceptive stimuli. In addition, Aδ fibers transmit touch sensation projecting to the neurons of the inner laminas III-IV (dark blue neuron). The Aβ fibers transmit tactile sensations and project to the tactile neurons, as the Aδ fibers. **B)** Changes occurring during neuropathic pain. The nerve injury produces adaptative changes on some C fibers, the up-regulation of $Nav1.8^{+}$ channels and TPRV1 in injured C and Aδ fibers, as well as the novel expression of α_1-adrenoceptor and α_2-adrenoceptor in uninjured Aδ and Aβ fibers. There is a phenotype switch of the tactile Aβ fibers that start to express substance P and calcitonin gene-related peptide (CGRP), such as the nociceptive fibers. In the DRG there is an enhancement of the NGF release that produces the sprouting of the sympathetic perivascular neurons arround the large fibers cell bodies producing an increased excitation. In the dorsal horn of the spinal cord, the nociceptive neurons (green) are overexcited by the increased release of substance P and glutamate for the C fibers and indirectly by the Aδ fibers. Another phenomenon that contributes to neuropathic pain is the lost of GABAergic and enkephalinergic interneurons (blue neuron) and/or the changes in the membrane potential that produces excitation in the nociceptive neurons (green) when are stimulated by GABA. Other important change that ocurrs is the sprouting of tactile Aβ fibers from deeper laminas to innervate the nociceptive neurons (green), causing the allodynia.

Research into temperature-sensitive excitatory ion channels also identified a cold and menthol-sensitive transient receptor potential (TRP) channel activated in the 8–28 °C range (Patapoutian et al. 2003) that is expressed in small-diameter DRG neurons (McKemy et al. 2002) and up-regulated after peripheral nerve injury (Wasner et al. 2004). This up-regulation seems to participate in the peripheral sensitization of cold-sensitive nociceptors located on C fibers, which results in the sensory phenomenon of cold (Wasner et al. 2004) and mechanical hyperalgesia (Price 2000). Experimental nerve injury also triggers the expression of functional α_1 and α_2-adrenoceptors on cutaneous afferent fibers, which could also participate in the peripheral sensitization (Price et al. 1998). The concept of a pathological adrenergic coupling between sympathetic postganglionic fibers and afferent neurons establishes the conceptual framework for the use of sympathetic antagonists in some pain processes, such as CRPS (Price et al. 1998).

Another peripheral mechanism that occurs during neuropathic pain and contributes to the sensitization is the collateral sprouting of primary afferent neurons. This means that the fibers of the primary afferent neurons spread in their vicinity and eventually establish new synapses. The induction of the sprouting is consequence of nerve grow factor (NGF) action at the level of DRG, where the levels of mRNA are increased after nerve injury (Sebert and Shooter 1993) (Figure 2). The sprouting of sympathetic neurons (noradrenergic perivascular sympathetic postganglionic axons) into DRG forms baskets around the large diameter neurons that do not transmit pain under physiological conditions. This sympathetic input could activate the neurons because the terminals of the sprouted neurons establish functional synapses-like structures with the cell bodies having as consequence the aberrant transmission of pain.

ii. Central mechanisms

Multiple changes also occur in the spinal cord dorsal horn and supraspinal structures during the development of neuropathic pain. Indeed, peripheral nerve injury leads to an increase in the general excitability of the spinal cord neurons. This hyperexcitability is shown by increased neuronal activity in response to noxious stimuli and expansion of neuronal receptive fields to other segments (Baron 2006). This phenomenon participates in the so-called central sensitization, which is mainly initiated and maintained by the activity of the pathologically sensitized C-fibres. These fibers sensitize spinal cord dorsal horn neurons by releasing glutamate, which acts on post-synaptic NMDA receptors, and substance P, which acts on neurokinin 1 (NK1) receptors (Baron 2006) (Figure 3). Several intracellular cascades contribute to this central sensitization at the

level of the spinal cord, in particular the mitogen-activated protein kinase (MAPK) (Ji and Woolf 2001).

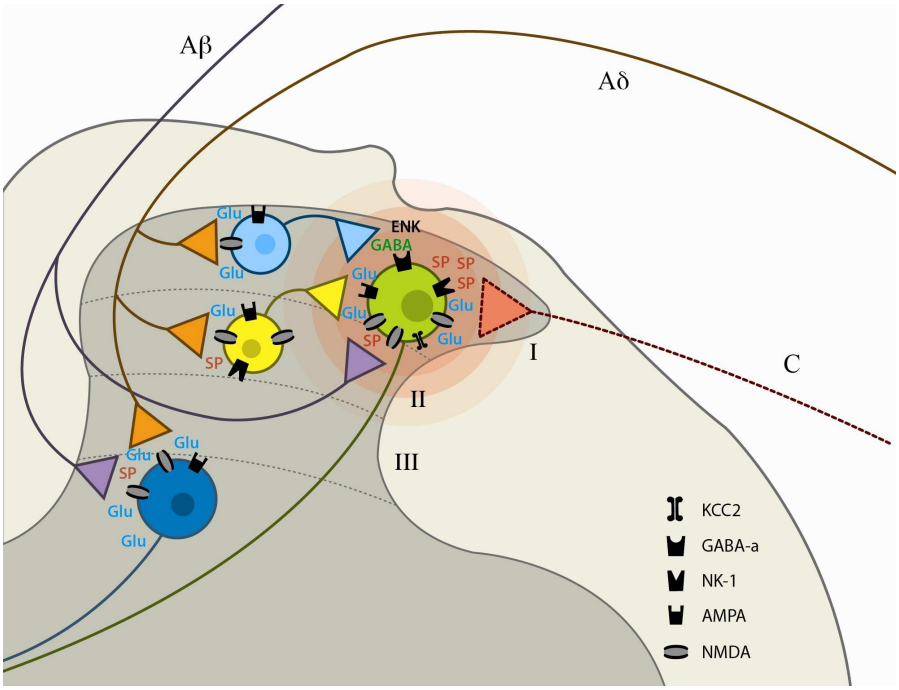

Figure 3. Spinal pathophisiology of neuropathic pain. The surviving injured C fiber terminals enhance the release of glutamate and substance P acting at the NMDA and NK-1 receptor, respectively, which increases the excitation of the nociceptive neuron (green). The injured Aδ fibers also increase the release of glutamate and substance P that act on the NMDA and NK-1 receptor of the excitatory interneurons (yellow neuron) increasing the release of the same neurotransmitters that stimulate again the nociceptive interneurons (green). These mechanisms are responsible of the hyperalgesia. A less clear mechanism is the loss of gamma aminobutiric acid (GABA) inhibitory tone. Aδ fibers made synapses with the GABAergic and enkephalinergic interneurons (blue), which release excitatory glutamate that excites the interneurons (blue). Some of that interneurons (blue) die as a consequence of the injury, and the survivors continue to release GABA and ENK to inhibit the excited nociceptive neurons (green). As a consequence of the injury, the expression KCC2 is down-regulated changing the membrane potential of the nociceptive neuron (green) and producing excitation when is stimulated by GABA. Finally, the structural change that produces the phenomenon of allodynia is the sprouting of the tactile Aβ fibers, which innervate the nociceptive interneurons (green). This Aβ sprouted fibers express glutamate and substance P that excites the nociceptive interneurons (green), once released and creates the loop that sends tactile stimulus from the body to the brain areas.

Another adaptive modification leading to the central sensitization is the reorganization of spinal neurons. The spinal reorganisation is a response to peripheral nerve injury of the Aβ fibers that sprout into lamina II of the dorsal horn, which is normally innervated by C fibers. At this level, the Aβ fibers establish functional synaptic contact with second order neurons that are involved in pain transmission (Scholz and Woolf 2002). As a consequence of these new synapses, low threshold non-noxious inputs from the Aβ fibers can be interpreted as nociceptive in origin although they are not (Bridges et al. 2001) (Figure 3). Furthermore, Aβ fibres suffer a phenotypic switch and begin to express nociceptors, substance P and calcitonin gene-related peptide (CGRP) that have an excitatory effect on postsynaptic neurons and potentiate the effects of substance P. All these nociceptors and neurokinins are normally expressed by primary afferent C fibers and Aδ fibers, but not in Aβ fibers (Miki et al. 1998). After central sensitization innocuous tactile stimuli become capable of activating spinal cord pain-signalling neurons via Aδ and Aβ low-threshold mechanoreceptors (Tal and Bennett 1994).

Changes in inhibitory pathways, such as reduction in the inhibitory control over dorsal horn neurons through different mechanisms, are also important in the central sensitization produced during neuropathic pain (Sugimoto et al. 1990; Woolf and Mannion 1999). Dorsal horn neurons receive a strong inhibitory input from gamma aminobutiric acid (GABA) releasing-interneurons. In rodents, peripheral nerve injury promotes a selective apoptotic loss of GABA-releasing inhibitory neurons in the superficial dorsal horn of the spinal cord (Moore et al. 2002), a mechanism that further increases central sensitization. Dorsal horn neurons receive a powerful descending modulating control from supraspinal brainstem centers, which has inhibitory as well as facilitatory effects (Vanegas and Schaible 2004). It was hypothesized that a loss of

function in descending inhibitory serotonin and noradrenalin pathways contributes to central sensitization during neuropathic pain (Ossipov et al. 2000a). In animals, mechanical allodynia after peripheral nerve injury depends on tonic activation of descending pathways that facilitate pain transmission, indicating that structures in the mesencephalic reticular formation, possibly the *nucleus cuneiformis* and the PAG are involved in central sensitization during neuropathic pain (Ossipov et al. 2000b). However, another alternative mechanism of intraspinal desinhibition following peripheral nerve injury has been proposed. This mechanism involves a trans-synaptic reduction in the expression of the potassium-chloride cotransporter (KCC) 2 in lamina I neurons, which disrupts anion homeostasis in these neurons. The resulting shift in the transmembrane anion gradient changes inhibitory anionic synaptic currents to be excitatory. The effect is that GABA release from normally inhibitory interneurons now paradoxically exerts an excitatory action on lamina I neurons, which also contributes to increase central sensitization (Coull et al. 2003) (Figure 3).

Most animal experiments investigating the mechanisms involved in central sensitization have been focused on the dorsal horn of the spinal cord. However, sensitized neurons are also found in the thalamus and primary somatosensory cortex after peripheral nerve injury in rodents (Guilbaud et al. 1992). Furthermore, magneto-encephalography, positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies demonstrate important changes in the somatosensory cortical representation and excitability in patients with phantom limb pain, CRPS type and central pain syndromes, (Flor et al. 1995; Pleger et al. 2004; Willoch et al. 2004; Maihofner et al. 2005), as well as in experimental pain models (Baron et al. 1999; Baron 2000)

Interestingly, these changes correlate with the intensity of the perceived pain and disappear after successful treatment of the painful symptoms (Maihofner et al. 2004; Pleger et al. 2005).

d. Effectiveness and limitations of available treatments

The current management of neuropathic pain includes non-pharmacological and pharmacological therapies. The surgical treatment and the conservative interventions can be included in the first category. However, there is not a fully effective treatment for palliating all symptoms of neuropathic pain in spite of the many approaches available.

Among the several non-pharmacological treatments available to attenuate the neuropathic pain symptoms, it is worth mentioning physical therapy, transcutaneous electric nerve stimulation, percutaneous electrical nerve stimulation, acupuncture or electropuncture, spinal cord stimulation, low-level laser therapy, superficial heat, ultrasonography, psychological-cognitive behavioural techniques and chiropractic procedures. Longitudinal studies of spinal cord stimulation have consistently shown significant pain relief in 50% to 60% of patients with neuropathic pain in legs or arms (Carter 2004). However, there are situations in which the only effective solution to treat neuropathic pain is surgery (Moulin et al. 2007). This is the case of traumatic peripheral nerve injury, lumbar disk hernia, or tumours, like neuromas, that can cause neuropathic pain. Surgical procedures include surgical release of entrapped nerve in traumatic peripheral injury, removal of prolapsed nucleus pulposus material of an intervertebral disc in lumbar hernia or surgical excision in the case of tumors.

Large systematic reviews of neuropathic pain treatment have shown that only 60%-70% patients achieved at least moderate pain relief after pharmacological treatment (Sindrup and Jensen 1999; Collins et al. 2000). Intolerable side-effects often limit the ability to achieve adequate pain control with a single agent, leading either to discontinuation of specific agents or to progressive treatment strategies to optimize pain control for individual patients (Namaka et al. 2004). Even within the same disease, responses to neuropathic pain treatment may vary from patient to patient. Most patients receive multiple drugs with divergent mechanisms of action that collectively work to diminish the peripheral and central manifestations of pain. In the last fifty years, the pharmacological treatment of neuropathic pain has included antidepressants, anticonvulsants, antiarrhytmics, topical local anaesthetics, capsaicin and, not without controversy, opioid analgesics (Banos et al. 2003). The evidences of effectivity of the available pharmacological treatments are summarized in the table 1.

However, drugs are only partially effective in relieving pain in most of patients. Complete relief is rarely achieved and a significant proportion of patients do not experiment enough analgesic effects with any of the available drugs. Therefore, the search for more effective and safe drugs is still imperative to improve the quality of life of neuropathic pain patients.

Table 1: Effectivity evidence of the available pharmacological treatments (modified from Attal and Finnerup, 2010).

Etiology	Level A Rating for Efficacy	Level B Rating for Efficacy	Level C Rating for Efficacy	Level A/B Rating for Inefficacy or Discrepant Results	Recommendations as First-Line Treatment	Recommendations as Second- or Third-Line Treatment
Diabetic neuropathy[1]	Duloxetine Gabapentin-morphine Gabapentin Oxycodone Pregabalin TCAs2 Tramadol alone or with acetaminophen Venlafaxine ER	Botulinum toxin* Dextrome-thorphan Gabapentin-venlafaxine* Levodopa*	Carbama-zepine Phenytoin	Capsaicin cream Lacosamide Lamotrigine Memantine Mexiletine Mianserin Oxcarbazepine SSRIs Topical clonidine Topiramate Valprate Zonisamide	Duloxetine Gabapentin Pregabalin TCAs Venlafaxine ER	Opioids Tramadol[3]
Postherpetic neuralgia	Capsaicin 8% patch Gabapentin Lidocaine plasters Opioids (morphine, oxycodone, methadone) Pregabalin TCAs[2]	Capsaicin cream Valproate*		Benzydamine (topical) Dextromethor-phan Fluphenazine Memantine Lorazepam Mexiletine Tramadol	Gabapentin Pregabalin TCAs Lidocaine plasters[4]	Capsaicin Opioids
Central pain[5]	Cannabinoids (oromucosal, oral) (MS) Pregabalin (SCI)	Lamotrigine (CPSP) TCAs (SCI, CPSP) Tramadol (SCI)* Opioids		Carbamazepine Gabapentin Duloxetine (found effective in allodynia in one study) Lamotrigine in SCI (except in patients with allodynia in one study) Levetiracetam Mexiletine S-ketamine iont. Valproate	Gabapentin Pregabalin TCAs	Cannabinoids (MS) Lamotrigine Opioids Tramadol (SCI)

Classification of evidence for drug treatments in commonly studied neuropathic pain conditions and recommendations for their use. Treatments are presented in alphabetical order. Only drugs used in repeated dosages and available or soon to be available for use are shown here (with the exception of treatments with long-lasting effects such as capsaicin patches). Drugs marked with an asterisk were found effective in single class II or III studies and are generally not recommended.
Abbreviations: iont.: iontophoresis; CPSP: central poststroke pain; ER: extended release; MS: multiple sclerosis; NK1: neurokinin 1; PHN: postherpetic
neuralgia; SCI: spinal cord injury; TCAs: tricyclic antidepressants.
[1] Only TCAs, tramadol, and venlafaxine were studied in nondiabetic neuropathies.
[2] TCAs include amitriptyline, clomipramine, nortriptyline, desipramine, and imipramine.
[3] Tramadol is recommended fi rst line in patients with acute exacerbations of pain, especially for the tramadol/acetaminophen combination.
[4] Lidocaine is recommended fi rst line mainly in elderly patients.
[5] Cannabinoids and lamotrigine are proposed for refractory cases.

3. Experimental study of neuropathic pain

a. General aspects

Animal models of neuropathic pain are essential to promote the research, to better understand the mechanisms underlying the pathophysiology of neuropathic pain and to design novel therapeutic strategies to obtain new compounds for clinical use (Bridges et al. 2001). Some animal models have shown to be predictive of the drug efficacy in patients, but there is a long history of failure at this respect. Unfortunately, experimental models give few clues about the clinical side effects of the drugs, and this has been one of the most important limitations with the new therapies. These considerations should be borne in the mind of researchers when working in experimental models of neuropathic pain.

b. Experimental models

The experimental animal models of neuropathic pain developed in the last three decades include both CNS and peripheral nerve system injuries. The last category incorporates many models due to the better accessibility, as those induced by mechanical injury (constriction, full ligation and section), physical methods (laser, cryogenics), metabolic disorders (diabetes), neurotoxicity (chemotherapeutic agents like paclitaxel and cisplatin), immunological Freund's adjuvant, tumour necrosis factor (TNF) and NGF stimulation. The main models are summarized in Table 2.

Among the available models, the most used are those that cause mechanical injury. They allow the study of the manifestations of neuropahic pain, the neurobiological mechanism involved and the efficacy

of possible pharmacological treatments. These models also share alterations in hind-paw cutaneous sensory thresholds following partial injury of a peripheral nerve, usually sciatic, as a common feature. Demonstration of hyperalgesia to noxious thermal stimuli and allodynia to cold and mechanical stimuli are currently used as outcome measures. The three most commonly used peripheral models are the chronic constriction injury (CCI) of sciatic nerve (Bennett and Xie 1988), the partial sciatic nerve ligation model (PSNL) (Seltzer et al. 1990; Malmberg and Basbaum 1998) and the spinal nerve ligation model (SNL) (Kim and Chung 1992).

The CCI model consists of the loose ligation of the sciatic nerve at mid-thigh level with chromic gut sutures (Bennett and Xie 1988). An inflammatory reaction develops in response to the catgut and consequently a loss of most A fibers and some C-fibers occurs, although few cell bodies are lost (Tandrup et al. 2000). This injury is associated with spontaneous pain-related behaviour, allodynia and hyperalgesia. A significant inflammatory component is associated to the development of the painful neuropathy since the CCI rats exposed to this procedure showed decreases thermal hyperalgesia after anti-inflammatory treatment (Wagner et al. 1998). In addition, there is a large degree of operator variability in this model, depending on differences in the tightness of the ligatures (Bridges et al. 2001).

The PSNL model also consists of injury to the sciatic nerve at mid-thigh level. In this model, a tight ligation is created around 33–50% of the sciatic nerve, leaving the rest of the nerve ‘uninjured’ (Seltzer et al. 1990; Malmberg and Basbaum 1998). This is associated with the development of spontaneous pain-like behaviour, allodynia and hyperalgesia. Although this model is regarded as having less inflammatory the PSNL injury to a specific DRG or level of the component than the CCI model, there is still

Table 2. Main neuropathic pain experimental models

Model	Reference
Central nervous system	
Posterior rhizotomy	Basbaum and Wall, 1991
Dysesthesic syndrome	Levitt, 1991
Laser induced ischemia of spinal cord	Hao *et al.,* 1991
Spinal neurotoxicity induced by quisqualic acid	Yerzierski and Park, 1993
Peripheral nervous system	
Mechanical injury	
Nerve section	Wall *et al.,* 1979
Chronic constriction injury of sciatic nerve	Bennet and Xie, 1988
Partial sciatic nerve ligation	Seltzer *et al.,* 1990
Spinal nerve ligation	Kim and Chung, 1992
Neuropathy induced by polyethylene cuffs on sciatic nerve	Mosconi and Kruger, 1996
Physical injury	
Peripheral cryogenic nerve lesion	DeLeo *et al.,* 1991
Laser induced ischemia of sciatic nerve	Kupers *et al.,* 1998
Metabolic neuropathy	
Sterptozotocin induced diabetic neuropathy	Ahlgren end Levine, 1993
Chemotherapy induced neuropathy	
Vincristine administration	Authier *et al.,* 1999
Paclitaxel administration	Polomano *et al.,* 2001
Cisplatin administration	Authier *et al.,* 2003
Neuroinflammation induced neuropathy	
Tumour necrosis factor administration	Wagner and Myers, 1996
Nerve growth factor administration	Ruiz *et al.,* 2004
Complete Freund's adjuvant administration	Eliav *et al.,* 1999

high variability depending on the number of ligated nerves per animal. In addition, it is not easy to relate spinal cord since usually a random mixture of L4 and L5 spinal nerve afferents are injured (Bridges et al. 2001).

The SNL model consists of injury to the L5 and L6 spinal nerves, which contribute to the sciatic nerve (Kim and Chung 1992). This injury is also associated with the development of spontaneous pain-like behaviour as well as long lasting allodynia and hyperalgesia. A tight ligation of only the L5 spinal nerve resulted in comparative symptoms to the L5 and L6 ligation group and hence some researchers now use this procedure as a modified SNL model (Bridges et al. 2001). SNL model allows examining cellular responses to the injury at the DRG level since the L5 and L6 DRGs are affected, whereas the L4 DRG is not (Li et al. 2000).

c. Use of genetically modified animals

One challenge of modern neurobiology is the identification of individual molecules that operate within neural circuits, regulate brain function and control synaptic plasticity. A unique approach to the molecular basis of neuronal activity consists in manipulating the genome of higher organisms. In this context, the mouse represents an exceptional animal model which is prone to targeted gene modifications, shares the complex genome and neuroanatomical organization of mammals, and can be studied in paradigms that model the wide array of human neurological and psychiatric diseases.

The advent of gene targeting technology by homologous recombination in mouse has led to a first generation of so called knockout (KO) animals (Capecchi 1989). In these mutant mice, the gene of interest is inactivated.

The caractreization of the phenotype of these null mutant mice has provided invaluable information in the identification of key proteins involved in neural development and plasticity, as well as neurotransmission or drug actions in vivo. Spatial and temporal control of the gene KO, generally referred to as conditional gene KO, was the next step in the development of gene targeting technologies.

Another genetical approach recently used is the overexpresion of a targeted gene using a plasmid. This plasmid contains a vector with the DNA sequence of the gene and a specific promoter. This transgenic tecnique permits the overexpression of a transcriptionally active or repressor gene in a specific tissue selecting the appropiate promoter (Watkins et al. 2003).

d. Advantages and limitations of transgenic experimental models

As explained before, genetically modified mice have been useful tools to better understand the mechanisms underlying the physiology of diverse biological process. Even that, genetically modified mice, either by increasing or eliminating a specific gene, may be limited by the fact that this genetic change may be affecting other biological components, perhaps participating in the effects evaluated in these genetic models. The conventional KO technology, however, has limited utility in several situations in the neurobiological studies. First, the gene of interest could be essential for development and survival, and the gene KO lead to a lethal phenotype. Second, the targeted gene could be important for normal development and share functional redundancy with other genes. In this case the phenotype could be hardly detectable and would likely result

from compensatory mechanisms that may be difficult to clarify. Third, some proteins are widely expressed both in the nervous system and peripheral tissues, and gene KO throughout the body does not address their specific role in cerebral function. Additionally, and because of the high anatomic complexity of the nervous system, many neural proteins have distinct functions depending on their site of expression within neurons and neural circuits. As a consequence, the complete deletion of a specific protein throughout the nervous system may prove ineffective towards understanding fine molecular processes in higher brain functions (Gaveriaux-Ruff and Kieffer 2007)

Pharmacological studies using selective ligands for the specific protein product of the modified gene would be useful to confirm the relevance of the results obtained with these animals. However, a compound specifically selective for a unique target is difficult to obtain, and many protein targets without a known drug ligand have also been identified. Moreover, the selectivity of a compound is in inverse relation to the dose, which could represent an important limitation for the interpretation of the results obtained with the pharmacological tools. Nevertheless, genetic manipulations have been considered a key approach to identify alterations associated to different pathological conditions and to the discovering of new potential therapeutic targets in a variety of neuropsychiatry disorders.

4. Endogenous opioid system

a. General aspects

The isolation and purification of the first alkaloid from opium, morphine, in 1806 by Friedrich Sertürner established the starting point for the modern pharmacognosy. For the first time, the main active principle of a plant was isolated and could be used in therapeutics. After this discovery, the pharmacology of natural substances advanced quickly with the identification and isolation of different plant compounds with a great spectrum of activities. This fact generated the possibility of using these compounds to investigate their effects, identify their mode of action, and use them both as therapeutic drugs in the medical practice and as chemical template to develop new drugs. At the end, this first discovery allowed the identification of opioid receptors almost one hundred and seventy years later.

In the decade of 1960s, it had become apparent that opioid drugs were likely to exert their actions at specific receptor sites (McClane and Martin 1967). These receptors were first identified as specific molecules through the use of binding studies, in which opiates that had been labelled with radioisotopes were found to bind to brain membrane homogenates (Goldstein et al. 1971). In 1973, the first experimental evidence of the existence of an opioid receptor was published by the Terenius group at Uppsala (Terenius 1973) that was followed shortly by the papers of two other groups, the first at Baltimore (Pert and Snyder 1973) and the second at New York (Simon et al. 1973). Only two years after, the first endogenous molecules that bind to that opioid receptor were discovered from the pig's brain and therefore were called enkephalins (Hughes et al. 1975). In 1976, it was discovered another endogenous peptide with

morphine-like action, which was named endorphin, an abbreviation of endogenous morphine (Simantov and Snyder 1976). In 1979, dynorphin was also discovered (Goldstein et al. 1979). Therefore, the two elements, receptors and neurotransmitters, of a new neurotransmission system at CNS were established.

This endogenous system consists of a series of families of opioid peptides that bind to different types of opioid receptors**.** Three different opioid receptors, μ (mu opioid receptor, MOR), ∂ (delta opioid receptor or DOR) and κ (kappa opioid receptor or KOR), have been identified and cloned (Kieffer 1995). Moreover, three families of opioid precursors responsible for the synthesis of the different opioid peptides have been identified and cloned (Kieffer 1995)

b. Biology of the opioid system

i. Opioid receptors

Three different types of opioid receptors have been cloned in experimental animals and humans: MOR, DOR and KOR (Kieffer 1999). Moreover, at least two subclasses of MOR (μ1, μ2) and DOR (δ1, δ2) and three subclasses of KOR (κ1, κ2, κ3) generated by post-transcriptional modifications have also been suggested (Minami and Satoh 1995), although the relevance of these subtypes has not been clarified yet. An additional event that enhances the complexity of opioid receptors is the formation of heterodimers, in particular the existence of heterodimers of type DOR / KOR and MOR / DOR. These variants and associations represent a new functional structure with different properties to the original receptor and could explain some of the pharmacological responses of opioids that are not consistent with the activation of classical

receptors (Jordan and Devi 1999, Gomes, 2000 #15099; Devi 2001). A new receptor, the nociceptin / orphanin (NOP, orphanin-receptor like 1 or ORL1), was initially proposed to be part of the opioid receptor family but it was considered to belong to an antiopioid system by the pharmacological actions arising from its activation (Anton et al. 1996).

Opioid receptors belong to the superfamily of G protein-coupled receptors characterized by the presence of seven transmembrane domains. Opioid receptors act primarily through the activation of G protein Gi / Go inhibiting adenylyl cyclase activity, and thereby reduce the levels of cyclic adenosine monophosphate (cAMP) and the activity of protein kinase A (PKA), which results in a decreased phosphorylation of intracellular effectors. Its activation also induces a change in the activity of the signaling pathways of phospholipase C and MAPK, such as ERK (Belcheva et al. 2001) These changes produce the activation of the transcription factors such as CREB at ELK-1 that leads to the transmition of the signal inside the nucleus and the transcription of genes (Ligeza et al. 2008). Because of their actions on these G proteins and intracellular signaling systems, opioid receptor stimulation produces an inhibition of voltage-activated Ca2+ channels and the stimulation of inwardly rectifying K+ channels, thereby reducing the release of neurotransmitters at presynaptic level and decreasing the excitability of the membrane potential. This leads to a hyperpolarization of the membrane and, consequently, to a reduction in neuronal activity. They are receptors that mainly mediate inhibitory actions (Gutstein and Akil 2001; Álvarez and Farré 2005).

Opioid receptors are broadly distributed in both the CNS and peripheral tissues as revealed by different autoradiographical studies (Mansour et al. 1995). The MOR are the opioid receptors with a wider distribution in the

brain, mainly in the structures related to nociceptive control, motor responses and motivation. These receptors are highly expressed in the nucleus caudate-putamen, *globus pallidus* and *ventral pallidum*, bed nucleus of the *stria terminalis*, thalamus, medial and cortical amygdala, interpeduncular nucleus, nucleus raphe and *locus coeruleus*, among other structures. In the striatum, these receptors are distributed in clusters or patches. Compared to the MOR, DOR have a more restricted distribution in the brain. They are mainly located in regions such as the neocortex, striatum (which have dense and homogeneous distribution), olfactory tubercle, *globus pallidus, ventral pallidum*, septal nucleus, amygdala, pontine nucleus and diagonal band of Broca. Finally, KOR have an even more restricted distribution than DOR and are primarily found in the striatum, olfactory tubercle, bed nucleus of the *stria terminalis*, dorsal and ventromedial hypothalamus, amygdala, PAG, nucleus raphe and *locus coeruleus* (Mansour et al. 1995).

In the spinal cord, approximately 60% of opioid receptors are MOR, while 21% are DOR and 19% KOR. MOR are mainly expressed in the gelatinous substance and to a lesser extent in other structures such as the *laminae* III, IV, V and VIII. The KOR, are also mainly expressed mostly in the *substantia gelatinosa*. The DOR are preferentially located in the lamina I of the dorsal horn. Most of MOR, DOR and KOR are expressed presynaptically in the spinal cord. However, they are also located postsynaptically on the plates of the ventral region of the spinal cord.

Opioid receptors are also expressed in peripheral tissues where they modulate various physiological functions. They are located at the level of sensory and sympathetic nerve fibers of the skin and joints, in the plexuses of the intestine, urinary bladder and vas deferens, in endocrine cells and in the immune system (Stein 1993).

The predominant involvement of each opioid receptor in the control of the different noxious stimuli seems to be related to the particular anatomical location of these receptors. Thus, MOR mediates mechanical, thermal and chemical high-intensity painful stimuli while DOR modulate thermal and chemical pain. However, it is not well known the precise nature of painful stimuli that are mediated by KOR (Yaksh 1993; Maekawa et al. 1994; Mansour et al. 1994; Schafer et al. 1994).

ii. Endogenous and exogenous ligands

Under physiological conditions, opioid receptors are activated by a group of compounds called endogenous opioid peptides. Currently there are three known precursors that give rise to three different families of these peptides: proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) (Álvarez and Farré 2005; Maldonado and Valverde 2005).

The main opioid peptide derived from POMC is the β-endorphin, which has high affinity for MOR and DOR. Opioid peptides derived from PENK are mainly met-enkephalin and leu-enkephalin that preferentially activates DOR and, to a lesser extent, MOR. These peptides differ only in a single amino acid (methionine or leucine) located at the N-terminal. Other opioid peptides derived from this precursor are peptide F, peptide I, peptide B and sinencephaline, among others (Hedna and Cassuto, 1987). The derivatives of PDYN are dynorphins (A and B) and neoendorphines (α and β) with preferential affinity for KOR, and leu-enkephalin that preferentially activated DOR. A new group of opioid peptides with high affinity for MOR has been described more recently (Zadina et al. 1997), which includes endorphins 1 and 2. However, the absence in the human

genome of the sequence of these peptides has questioned their possible existence as endogenous molecules. The anti-opioid peptide derived from the pronociceptin / orphanin FQ is the nociceptin / orphanin Q, which has high affinity for the nociceptin / orphanin receptor. The pharmacological properties of this peptide are, in general, opposite to those induced by classical opioid peptides (Minami and Satoh 1995, Maldonado and Valverde, 2005; Civelli 2008).

Table 3. Exogenous opioid ligands and their main pharmacological actions (Álvarez and Farré 2005)

Compound/receptor	MOR	DOR	KOR
Morphine	Ag +++	Ag +	Ag +
Heroin	Ag +++	Ag +	Ag +
Methadone	Ag +++	Ag +	
Fentanyl	Ag +++	Ag +	
Pentazocine	An -	Ag +	Ag +++
DPDPE	Ag +	Ag +++	
Deltorphine I		Ag +++	
SCN80		Ag ++	
Salvinorin A			Ag +++
U-50, 488			Ag +++
Buprenorphine	Ag +++		An - -
Naloxone	An - - -	An - -	An -
Naltrexone	An - - -	An - -	An -
Naltrindol		An - - -	
Nor-binaltorphimine			An - - -

Sigma (σ) receptors were first defined as a subclass of opioid receptors (Skuza and Wedzony 2004). However, today they are considered as unique binding sites with their own brain function different from the opioid receptors (Skuza and Wedzony 2004). Two sigma receptors

subtypes have been well characterized at the present moment: σ-1 and σ-2 receptor (Hellewell and Bowen 1990; Quirion et al. 1992; Bowen 2000). Recently, it has been discovered that N,N-dimethyltryptamine (DMT) is the endogenous ligand to σ receptors (Fontanilla et al. 2009).

Exogenous ligands can be classified into natural, semisynthetic or synthetic depending on its origin. These drugs may be categorized as agonists (Ag), antagonists (An) and partial agonists depending on the nature of their interaction with opioid receptors. Table 3 summarizes the main exogenous opioid ligands and their actions.

c. Opioid system and pain modulation

The endogenous opioid system is involved in several physiological responces that are essential for the survival of species, such as responses to pain and stress, the control of motivation and reinforcement, and motor and homeostatic adaptive functions including food intake and regulation of body temperature. Endogenous opioid system also contributes to the control of some autonomic nervous system functions, such as breathing and gastrointestinal motility. In addition, it also participates in the modulation of immune-based responses (Olson and Welch 1991; Bodnar and Hadjimarkou 2003).

The endogenous opioid system plays a crucial role in the inhibitory control of pain (Mogil et al. 2000). Due to the wide distribution, it modulates the nociception at both peripheral nervous system and CNS. At the peripheral level and during the inflammation process, the immune cells synthesize and release opioid peptides that bind opioid receptors of the peripheral nerve terminals in the inflamed tissue. As a consequence,

nerve excitability and release of inflammatory mediators are reduced by this opioid-mediated response (Rittner et al. 2008).

The endogenous opioid system also regulates the nociceptive pathways in the CNS, both at the level of the spinal cord and the supraspinal centres, and also by activating the descending inhibitory pathways. The first central site of action is at the spinal level, where it inhibits the nociceptive transmission conveyed of Aδ and C fibers. The opioid receptors are expressed presinaptically in the terminals of these fibers and modulate the release of excitatory molecules like substance P, glutamate and CGRP (Minami and Satoh 1995; Flórez 2007). The opioid receptors are also located postsinaptically at spinal neurons like those that integrating the spinotalamic pathway that transmits nociception stimuli to supraspinal centers (Figure 4).

At supraspinal level the opioid receptor and peptides are expresed in amygdala, thalamus, hipothalamus, cerebral cortex and PAG matter (Mansour et al. 1995). The endogenous opioid system inhibits the nociceptive transmission of the ascendant pathway that innervates the thalamus.

In addition, the action on the thalamic projections to the hypothalamus inhibits the vegetative component of pain (Serrano-Atero et al. 2002; Flórez 2007). Thalamic projections to the cortex are under the control of the endogenous opioid system. In these two areas, the nociceptive information is consciously integrated. The cortex defines the particular characteristics, intensity and localization of the pain sensation. Moreover, the opioid receptors are abundantly expressed in the limbic system inhibiting the emotional perception of pain (Guyton 1997; Flórez 2007). All these evidences show that the endogenous opioid system acts

decreasing the ability to integrate nociceptive information and therefore, reducing the emotional component of pain.

Finally, the endogenous opioid system also operates in the descending control of pain. In this system there are different cells ("on cells" and "off cells") that modulate the input of nociceptive information to the upward pain transmission. "On cells" facilitate nociceptive transmission, whereas "off cells" inhibit it. The endogenous opioid system regulates the activity of these cells inhibiting "on cells" and activating "off cells" (Minami and Satoh 1995; Guyton 1997; Flórez 2007). Thus, many neurons in the PAG matter release enkephalins, dynorphins and β-endorphin, in addition to connect synaptically with hypothalamic projections rich in β-endorphin (Guyton 1997; Serrano-Atero et al. 2002). Enkephalinergic neurons in the PAG synapse with serotonin raphe magnus nucleus that project to the dorsal horn of the spinal cord. In turn, they act on enkephalinergic neurons present at the dorsal horn where it is believed that the enkephalin produces presynaptic inhibition of Aδ and C fibers entering the spinal cord. In addition, noradrenergic terminals that depart from the *locus coeruleus* and project into the dorsal horn are also regulated by the endogenous opioid system (Serrano-Atero et al. 2002).

Besides the antinociceptive activity dynorphin may have in certain situations pronociceptive effects at the spinal level. Thus, the increase of this peptide at the spinal level following chronic treatment of either opioid or cannabinoid agonists (Vanderah et al. 2000; Gardell et al. 2002) has been linked with the development of hyperalgesia and allodynia (Laughlin et al. 2001). This pronociceptive action appears to be due to activation of NMDA receptors by dynorphins (Laughlin et al. 2001).Thus, spinal dynorphins in turn causes an increased release of excitatory

neurotransmitters and substance P, an action that contributes to amplify pain transmission (Ossipov et al. 2003) (Figure 4).

d. Opioid therapy and neuropathic pain

The endogenous opioid system participates in the physiological control of the different nociceptive stimuli through the activation of the opioid receptors (Martin et al. 2003). Agonists of MOR have been largely used in clinic to treat severe pain associated to different conditions. In spite of the fact that these drugs are the first-line treatment of many acute and chronic pains, they have a limited effectiveness in the treatment of neuropathic pain in humans (Arner and Meyerson 1988) and animal models (Ossipov et al. 1995).

The limited effectiveness of opioids in neuropathic pain can be related to the adaptative changes reported in the opioid system during this pathological state. A decrease of MOR mRNA in the ipsilateral dorsal root ganglion and lumbar spinal cord has been found (Pol et al. 2006). An increased activity in spinal cord of the endogenous anti-opioid cholecystokinin was also found under neuropathic pain conditions (Wiesenfeld-Hallin and Xu 2001), as well as an enhancement in the spinal dynorphin content as a consequence of peripheral nerve injury in rats and mice (Bian et al. 1999; Wang et al. 2001). It was demonstrated by using PDYN KO mice exposed to L5/L6 spinal nerve ligation (Wang et al. 2001) or partial sciatic nerve injury (Xu et al. 2004) that dynorphin is essential in the maintenance of neuropathic pain state. Dynorphin has dual opposite effects in the pathophysiology of neuropathic pain: antinociceptive properties acting at KOR (Xu et al. 2004) and pronociceptive effects acting on NMDA receptors, which participates in the sensitization of the spinal cord (Bian et al. 1999; Wang et al. 2001)

(Figure 4). DOR and KOR densities in lumbar spinal cord did not change after spinal nerve ligation (Wang et al. 2001).

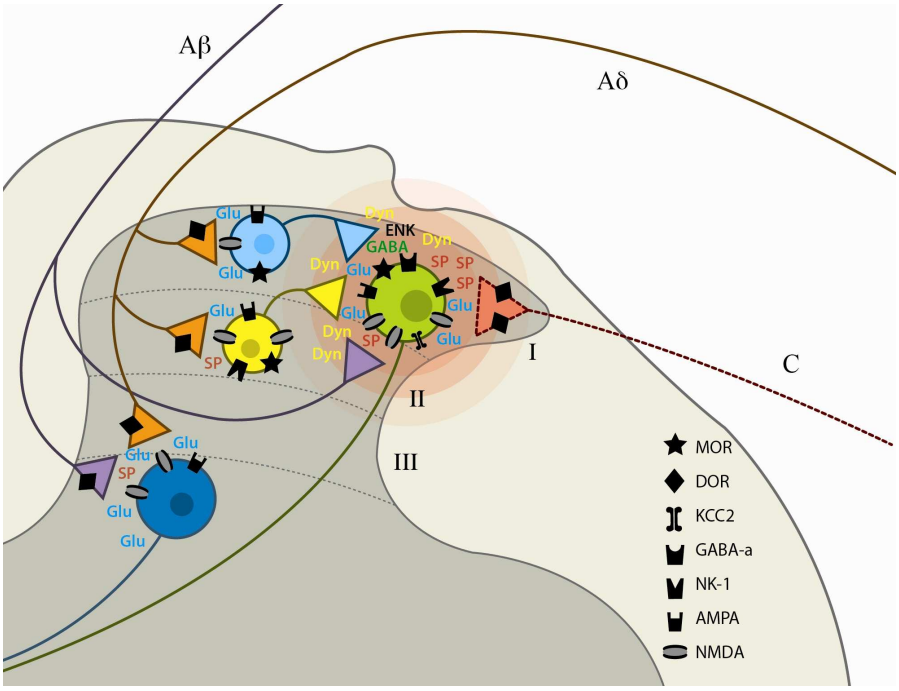

Figure 4. Implication of endogenous opioid system in the neuropathic pain pathophysiology. One of the implications of endogenous opioid system in the development and maintenance of neuropathic pain is the enhanced release of DYN by the glutamatergic/ substance P excitatory interneuron (yellow) and the GABAergic/ENKergic inhibitory interneuron (blue) overexcited by the glutamate and substance P of the injured Aδ fibers. This enhanced release of DYN acts mainly on the NMDA receptor of the nociceptive neuron (green) producing the hyperpolarization and overexcitation that produces finally the phenomenon of hyperalgesia and allodynia. This depends of the fiber type that stimulates the nociceptive neuron as showed in the figure 3. The presence of postsynaptic MOR in the nociceptive neuron (green) and in the glutamatergic/substance P excitatory interneuron (yellow) can inhibit the noxius ascendent stimuli acting directly or indirectly at the nociceptive neuron. The activation of MOR in the GABAergic/ENKergic inhibitory interneuron (blue) stops the release of GABA and ENK reducing the inhibitory tone that modulates the nociceptive neuron

(green). If we consider that, as consequence of the injury in the nociceptive neuron (green), down-regulates the expression KCC2. The consequence is a change in the membrane potential which produce an excitation when is stimulated by GABA. A reduction of GABA release can diminish the overexcitation of the nociceptive neuron (green). Finally, the presence of presynaptic DOR in the terminals of aferent fibers and in some interneurons can reduce the release of glutamate and substance P from the afferent injured terminals. In turn, this modulates the overexcitation of the nociceptive neuron (green) reducing the manifestations of neuropathic pain.

Several findings suggest that DOR could represent an interesting target for the treatment of neuropathic pain (Mika et al. 2001). Indeed, these receptors play an important role in the nociceptive control at the spinal cord (Mattia et al. 1992) and several studies have suggested that DOR could counteract the development of neuropathic pain symptoms (Table 4). There are many pharmacological studies using DOR agonists that reveal the analgesic effect of these compounds in diverse animal models and species (Table 4). The activation of DOR produces neuron hyperpolarization in C fibers and inhibits the excitatory glutamatergic synaptic transmission in the spinal dorsal horn (Glaum et al. 1994), thereby inhibiting the ascending noxious stimuli (Figure 4). Contradictory results have been published in reference of the expression of DOR in the areas related to pain transmission. A down-regulation of these receptors has been shown using immunohistological techniques after nerve injury in some models of neuropathic pain in areas related to the control of nociceptive transmission, including the spinal cord (Robertson et al. 1999; Stone et al. 2004). In contrast, an up-regulation of DOR protein was found in DRG of rodents exposed to neuropathic pain compared to sham, that appeared to be due to enhanced expression in both small and large DRG neurons (Kabli and Cahill 2007). Another study reported no significant change in DOR mRNA levels in the mouse DRG following partial nerve

ligation (Pol et al. 2006). The functional consequences of these changes remain to be clarified, as well as the specific role of DOR in the pathophysiology of the neuropathic pain.

Other mechanisms could be involved in the limited effectiveness of the classical opioids used in clinic for the treatment of neuropathic pain. The adaptative changes leading to pain transmition by Aδ and Aβ fibers during neuropathic pain conditions (explained in the chapter 2. C. i. and ii), could participate in this limited effectiveness considering the absence of opioid receptors in these large diameter fibers. In addition, CCK-mediated changes in the descending modulatory pathways appear to contribute to neuropathic pain and opioid-induced tolerance and hyperalgesia (King et al. 2005). There also evidence that opioid-induced tolerance and hyperalgesia share common cellular mechanisms that are related to changes in NMDA receptors (Mao et al. 1994; Mao et al. 2002) as equally occurs in the pathophysiology of neuropathic pain, which could contribute to the ineffectiveness of classical opioids.

Besides all these considerations, the effectiveness of opioids extends from nociceptive to neuropathic pain states (Smith 2008). Although neuropathic pain does not respond reliably, randomized clinical trials have shown a beneficial effect of opioids in several neuropathies (Banos et al. 2003), although frequent and severe side effects can limit their use in some patients. Moreover, the potential development of tolerance and dependence may complicate the use of these drugs in patients with non-cancer chronic pain and may represent in some particular cases an additional limitation for the clinical use of opioids (Benyamin et al. 2008).

Table 4: Analgesic effects of delta opioid agonists in mice, rats and monkeys exposed to inflammatory, cancer and neuropathic pain models (Gaveriaux-Ruff et al.)

Mice

Pain model	Agonist	Effective dose	Route	Assay	Modality	Reference
Formalin phase 2	SNC80 Tan-67 Compound 46 Morphine	79 umol/kg 52 umol/kg 10 umol/kg 69 umol/kg	iv#			(Barn et al. 2001)
	Deltorphin	10 ug	it			(Morinville et al. 2003))
Cancer pain	DPDPE DAMGO	30 ug 0.6 ug	Peri-tumoral	Hot plate	H	(Baamonde et al. 2005)
	DValAla-Enk Morphine	1.3 mg/kg 5.3 mg/kg	ip ip	Von Frey	M	(Brainin-Mattos et al. 2006)
Diabetic pain	Tan-67 (-)	6.2 ug	icv	Tail flick	H	(Kamei et al. 1997) *
Inflammatory pain	SNC80 Deltorphin	45 ug 0.72 ug	it it	Plantar test	H	(Gendron et al. 2007a)*
	DPDPE	100 nmol	ipl	Plantar test	H	(Hervera et al. 2009)
	SNC80	10 mg/kg	ip	Von Frey Plantar test	M H	(Gaveriaux-Ruff et al. 2008)
	SNC80 SNC80 AR-M100390 AR-M100390	10 mg/kg 10 mg/kg 	ip po 	Von Frey Tail immersion Von Frey Tail immersion	M H M H	(Pradhan et al. 2009)
	SNC80	10 nmol	it	Von Frey	M	(Scherrer et al. 2009)
Neuropathic pain	SNC80	10 nmol	it	Von Frey	M	(Scherrer et al. 2009)
	DPDPE	86 nmol 76 nmol 115 nmol	ipl ipl ipl	Plantar test Von Frey Cold plate	H M C	(Hervera et al. 2010)

Rats

Pain model	Agonist	Effective dose	Route	Assay	Modality	Reference
Formalin phase 2	Deltorphin Morphine Deltorphin Morphine	20 nmol 10 nmol 100 nmol 100 nmol	it it ipl ipl			(Bilsky et al. 1996)
	Deltorphin	32 ug	it			(Cahill et al. 2001)
	Deltorphin	10 ug	it			(Pradhan et al. 2006)
	SNC80 DSLET DAMGO Morphine	26 nmol 19 nmol 1 nmol 108 nmol	it			(Obara et al. 2009)
Inflammatory pain	SNC80 Deltorphin Morphine	120 nmol 11 nmol 5.6 nmol	icv	Plantar test	H	(Fraser et al. 2000)*
	SB-235863 Morphine	7.5 mg/kg 1.9 mg/kg	po	Plantar test	H	(Petrillo et al. 2003)*
	Deltorphin	0.06-0.6 ug	intra-RVM	Plantar test	H	(Hurley and Hammond 2000)*
	SNC80 Morphine	50 mg/kg 1.3 mg/kg	sc	Plantar test	H	(Gallantine and Meert 2005)
	Deltorphin	0.4 ug	it	Plantar test	H	(Cahill et al. 2003)*
	Deltorphin	10 ug 10 ug	it	Plantar test Tail immersion	H H	(Gendron et al. 2007a)*
	SNC80	5 umol/kg	intra-arterial	Flexor reflex	M	(Cao et al. 2001)
	SNC80	40 ug	ipl	Paw pressure	M	(Pacheco and Duarte 2005)
	DPDPE DAMGO	40 ug 1 ug	ipl	Paw pressure	M	(Stein et al. 1989)*
	DPDPE DAMGO	50 ug 8 ug	ipl	Paw pressure	M	(Zhou et al. 1998)
	NIH 11082	30 mg/kg	sc	Paw pressure	M	(Aceto et al. 2007)
	ADL5859	1.4 mg/kg	po	Paw pressure	M	(Le Bourdonnec et al. 2008)
	ADL5747	0.03 mg/kg	po	Paw pressure	M	(Le bourdonnec et al. 2009)
	JNJ-20788560	7-13 mg/kg	po	Plantar test	H	(Codd et al.

		0.3 mg/kg		Paw pressure	M	2009)*
	Compound 8e	17 umol/kg	iv	N.I.		(Jones et al.
	Morphine	3 umol/kg	iv	N.I.		2009)
Neuropathic	SB-235863	10 mg/kg	po	Plantar Test	H	(Petrillo et al. 2003)*
pain	Deltorphin	15 ug	it	Cold plate	C	(Mika et al. 2001)
	Deltorphin	10 ug	it	Von Frey	M	(Holdridge
		10 ug		Cold plate	C	and Cahill 2007)*
	Morphine	10 ug	ipl	Von Frey	M	(Kabli and
	Deltorphin	50 ug				Cahill 2007)
	SNC80	54 nmol	ipl	Von Frey	M	(Obara et al.
	DSLET	23 nmol				2009)
	DAMGO	5.6 nmol				
	Morphine	554 nmol				
	SNC80	111 nmol	ipl	Plantar test	H	
	DSLET	111 nmol				
	DAMGO	9.5 nmol				
	Morphine	1056 nmol				
	JNJ-20788560	100 mg/kg	po	Von Frey	M	(Codd et al. 2009)*

Monkeys

Pain model	Agonist	Effective dose	Route	Assay	Modality	Reference
Hyperalgesia						
induced by						
Capsaicin	SNC80	3.8 mg/kg	sc	Tail immersion	H	(Brandt et al.
PGE2[#]		0.8 mg/kg				2001)
CFA		0.56 mg/kg				

Analgesic effects of several selective delta agonists *in vivo*. Analgesic effects of SNC80 and other delta agonists (deltorphin, DPDPE, DSLET) are also listed when their selectivity of action for DOR were assessed either by delta selective antagonists, KO, anti-sense or interfering RNA. The analgesic effects of MOR agonists are also indicated when these were compared to those of delta agonists in the cited publications, for comparison purposes. Formalin phase 2 response (model for early inflammation) was induced by injection of formalin into the hindpaw. Cancer pain was induced by injection of NCTC 2472 osteosarcoma cells (Baamonde et al. 2005; Brainin-Mattos et al. 2006). Diabetic pain was induced by streptozotocin injection. Inflammatory pain was induced by injection of Complete Freund's Adjuvant (CFA) in the majority of studies, and by zymosan (Codd et

al. 2009), yeast (Codd et al. 2009), prostaglandin-E2 (PGE2) (Pacheco and Duarte 2005), or carrageenan (Petrillo et al. 2003; Jones et al. 2009). Neuropathic pain was induced by crushed sciatic nerve (Mika et al. 2001), partial sciatic nerve ligation (Petrillo et al. 2003), cuffing of the sciatic nerve (Kabli and Cahill 2007), chronic constriction injury (CCI) (Holdridge and Cahill 2007; Kabli and Cahill 2007; Obara et al. 2009; Hervera et al. 2010), spinal nerve ligation (Codd et al. 2009) and spared nerve injury (Scherrer et al. 2009). In some reports (*), delta agonists were shown effective at injured ipsilateral side but not at at contralateral side nor before the induction of chronic pain. # abbreviations : DAMGO, d-Ala2,N-Me-Phe4-Gly5ol-enkephalin mu opioid agonist, DPDPE, (2-D-Penicillamine, 5-D-Penicillamine)-enkephalin; icv, intracerebroventricular, im, intramuscular ; ip, intraperitoneal ; ipl, intraplantar ; it, intrathecal ; N.I.. not indicated ; po, per os ; RVM rostral ventral medulla; sc, subcutaneous.

5. Endocannabinoid system

a. Recent historical aspects

The beginning of modern pharmacology of *Cannabis sativa* dates back to 1964 when Gaoni and Mechoulam were able to isolate and synthesize the main active ingredient in marijuana, delta-9-tetrahydrocannabinol (Δ 9-THC) (Gaoni and Mechoulam 1964). Δ 9-THC is of lipid nature and initially it was proposed that his actions were due to its interaction with the cell membrane and the subsequent alteration of the membrane fluidity. Over the years, research studies have described the physiological effects of cannabinoids (Mendelson et al. 1976; Jones 1978). From the 1980's, the synthesis of some synthetic cannabinoids was the key element to the discovery of the main receptor involved in the psychoactive effects of cannabinoids, which is now known by the name of CB_1 receptor (Devane et al. 1988). The CB_1 receptor was discovered in 1990 by Matsuda's group (Matsuda et al. 1990). This first discovery opened a wide field for research, which applied the same principle of reasoning that in the case of opioid receptors. The identification of the cannabinoid receptor suggested the existence of an endogenous ligand and a whole undiscovered new neurotransmitter system. In 1992, researchers from Mechoulam's laboratory discovered the first endocannabinoid, arachidonylethanolamide, which they called anandamide (Devane et al. 1992). Ananda is a Sanskrit word which means peace, so "anandamide" would be our inner "peace amide". In 1993, Munro and his colleagues discovered the second cannabinoid receptor, CB_2 receptor (Munro et al. 1993) and the following year the laboratory of Di Marzo elucidated the mechanism involved in the biosynthesis of anandamide (Di Marzo et al. 1994). A second endocannabinoid, 2-araquinodylglycerol (2-AG) was

isolated and short time later described (Mechoulam et al. 1995)., Various components of the endocannabinoid system were identified with these initial discoveries, although some important information, such as the carrier and the degrading enzymes was still unknown. In 1996, Cravatt *et al*. found that the fatty acid amide hydrolase (FAAH) was the enzime responsible for the degradation of the anandamide (Cravatt et al. 1996) and Stella found that the enzyme monoacylglycerol lipase (MAGL) hydrolyzes 2-AG (Stella et al. 1997). These findings defined many potential drug targets, which could modulate the activity of the endocannabinoid system. At this point, the main elements that integrate the endocannabinoid neurotransmission system were already known, but many issues where not yet clarified, such as, the precise mechanism of neurotransmission, the possible existence of other receptors or the therapeutic applicability of cannabinoid substances that act on them.

b. Biology of the endocannabinoid system

The endocannabinoid system has unique characteristics differing from other neurotransmitter systems. First, the endocannabinoids act as neuromodulator that inhibit the release of other neurotransmitters, such as GABA and glutamate. The endocannabinoids are retrograde neurotransmitters that are released from the postsynaptic neuron. The postsynaptic neuron in response to a stimulus synthesizes and releases the endocannabinoids in the synaptic cleft that stimulate the cannabinoid receptors on the presynaptic neuron, which inhibits the release of neurotransmitters (Wilson and Nicoll 2002).

Thus, the membrane depolarization and activation of metabotropic glutamate receptor (mGluR) seem to be the main stimuli that induce the

endocannabinoid synthesis. Both stimuli produce an influx of Ca^{2+} into the cell that stimulates endocannabinoid synthesis, anandamide or 2-AG depending on the type of neuron. The endocannabinoids act on the presynaptic neuron acting as retrograde inhibitory signals, and inhibit the presynaptic neuron, as indicated by the location of the CB_1 receptor. The CB_1 receptor activation decreases the permeability of Ca^{2+} channels, and enhances the permeability of K^+ channels (Pertwee 2005) (Figure 5). In addition, the endocannabinoids are not located in synaptic vesicles, they are synthesized on demand from the membrane phospholipids (Di Marzo et al. 1994), and immediately released in the synaptic cleft.

Several specific mechanisms of retrograde signaling mediated by endocannabinoids have been reported in various brain structures. In the hippocampus the depolarization of pyramidal neurons in CA1 layer temporarily inhibit GABA incoming synapses. This phenomenon is called suppression of depolarization-induced inhibition (DSI). The entry of Ca^{2+} in the postsynaptic neuron initiates the process by inducing the voltage changes in the body and dendrites of the neuron. The increasing levels of Ca^{2+} also initiate the synthesis of endocannabinoids in the postsynaptic neuron. This activation produces the inhibition of the release of GABA by the interneurons stopping the activity of the inhibitor (Willson and Nicoll, 2001). This retrograde signaling and its modulation is mediated by the endocannabinoid 2-AG (Stella et al. 1997). CB_1 agonists are able to mimic the DSI, while CB_1 antagonists block it (Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001), and DSI is abolished in CB_1 receptor KO mice (Katona et al. 1999; Yoshida et al. 2002). Many of the GABAergic neurons involved in the DSI phenomenon express CB_1 receptors in axon terminals. Outside the hippocampus, the endocannabinoid-mediated DSI has also been reported at the synapses of Purkinje cells and GABAergic interneurons of the cerebellum

(Maccarrone et al. 1998; Ohno-Shosaku et al. 2001; Trettel and Levine 2003) (Figure 5).

Neurons of the hippocampus and cerebellum have another mechanism of endocannabinoid-mediated retrograde signaling that also affects glutamate activity and is called suppression of depolarization-induced excitation (DSE) (Kim et al. 2002) (Figure 5).

i. Cannabinoid receptors

The two main receptors that conforms the endocanabinoid system are the CB_1 and CB_2 cannabinoid receptors. Both are G-protein coupled receptors with seven trasmembrane domains associated to the inhibitory Gi/o protein (Childers and Deadwyler 1996). Nevertheless, compelling evidence supports the existence of additional G protein coupled receptors with cannabinoid activity (Brown 2007). Recently, it has been accepted that the orphan receptor GPR55 can be considered as the third receptor with cannabinoid activity (Baker et al. 2006; Ryberg et al. 2007).

CB_1 receptors are the most abundantly expressed metabotropic receptors in the brain and their distribution has been well characterized both in rodents (Herkenham et al. 1991; Tsou et al. 1998) and humans (Westlake et al. 1991). CB_1 receptors are highly expressed in hippocampus, basal ganglia (caudate-putamen, *globus pallidus*, ectopeduncular nucleus and *substantia nigra*), cortex and cerebellum (Compton et al. 1990).

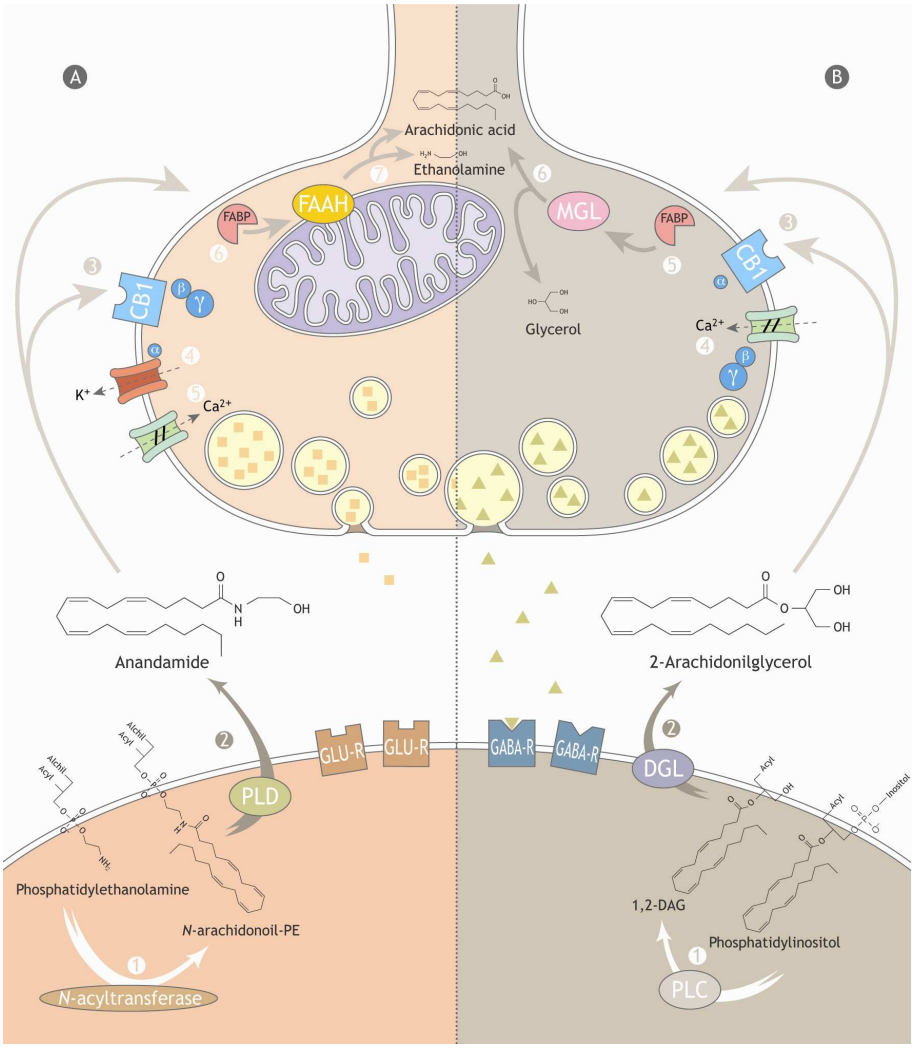

Figure 5. Retrograde mechanism of action of endocannabinoids. A) DSE in cerebellar neurons. Depolarization by calcium influx stimulates the biosynthesis of anandamide that occur in two steps: (1) Acylation of phosphatidylethanolamine with arachidonic acid catalyzed by N-acyltransferase. (2) Catabolism of N-arachidonoyl-PE by PLD to release anandamide. This anandamide traverse the synaptic cleft to activate postsynaptic CB_1 receptors (3). This is provoked by the α subunit of the G protein opening K^+ channels (4), hyperpolarizing the neuron and causing decreased influx of Ca^{2+} (5), with the consequent inhibition of glutamate release. The remaining anandamide difuses into the cell and it is arrested by the Faty Acid Binding Protein (FABP) (6) which transports it to the FAAH, and there is downgraded to AA and ethanolamine (7). B) DSI in GABAergic interneurons. Depolarization by calcium influx, produces 2-AG biosynthesis. The main route has two steps: (1) Catabolism of

phosphatidylinositol to 1,2-DAG by PLC. (2) Catabolism of 1,2-DAG to 2-AG by DGL. This 2-AG traverses the synaptic cleft to activate postsynaptic CB_1 receptors (3). This leads through γ β subunits of the G protein closing the Ca^{2+}channels (4), causing inhibition of GABA release. The remaining 2-AG diffused inside the cell is transported by the FABP into the presence of MGL, (5) and there is degraded to AA and glycerol by the MGL (6) (Adapted from Nadal and Baños, 2006).

CB_1 receptors are less expressed in the amygdala, hypothalamus, *nucleus accumbens*, thalamus, periapeduncular gray mater and the spinal cord as well as in other brain areas mainly in the telencephalon and diencephalum (Tsou et al. 1998; Cota et al. 2003a). In all these areas, CB_1 receptors are mainly expressed in GABAergic and glutamatergic neurons (Rodriguez et al. 2005). CB_1 receptors are also expressed in several peripheral organs. Thus, they are present in adipocytes (Cota et al. 2003b), liver (Osei-Hyiaman et al. 2006), lungs, smooth muscle, gastrointestinal tract (Calignano et al. 1997), pancreatic ß-cells (Bermudez-Silva et al. 2008), vascular endothelium (Liu et al. 2000), reproductive organs (Gerard et al. 1991), immune system (Kaminski et al. 1992; Galiegue et al. 1995), sensorial peripheral nerves (Hohmann and Herkenham 1999) and sympathetic nerves (Ishac et al. 1996).

The distribution of CB_2 receptors is quite different, and mainly restricted to the periphery in the immune system cells, such as macrophages, neutrophils, monocytes, B-lymphocytes, T-lymphocytes and microglial cells (Matsuda et al. 1990; Munro et al. 1993; Galiegue et al. 1995). Recently, CB_2 receptor expression has also been shown in skin nervous fibers and keratinocytes (Stander et al. 2005), bone cells such as osteoblasts, osteocytes and osteoclasts (Ofek et al. 2006), liver (Julien et al. 2005) and somatostatin secreting cells in pancreas (Bermudez-Silva et al. 2008)). The presence of CB_2 receptors has also been demonstrated at the CNS, in astrocytes (Sanchez et al. 2001), microglial cells (Walter et al.

2003; Nunez et al. 2004) and brainstem neurons (Van Sickle et al. 2005). In spite of the presence of mRNA of CB_2 receptor in cell cultures of Purkinje and granular cells of mice cerebellum (Skaper et al. 1996), and the staining with CB_2 antibody of murine and human neurons (Anand et al. 2008; Onaivi et al. 2008), the presence of functional CB_2 receptors in neurons is still a controversial issue. Recent evidences suggesting that CB_2 receptor mediates emotional behaviours, such as schizophrenia, anxiety, depression, memory and nociception support the presence of neuronal CB_2 receptors (Busquets-Garcia et al. ; Garcia-Gutierrez and Manzanares ; Ortega-Alvaro et al.).

Stimulation of CB_1 and CB_2 cannabinoid receptors activates a number of signal transduction pathways mainly via Gi/o family of G proteins. Activation of CB_1 and CB_2 cannabinoid receptors produces the inhibition of adenylate cyclase, and the corresponding decreased activity of the PKA pathway. The activation of cannabinoid receptors also stimulates the activity of the MAPK pathway. Cannabinoid receptor activation, through the stimulation of Gi/o proteins, is also directly coupled to inhibition of voltage-activated Ca^{2+} channels and stimulation of inwardly rectifying K^+ channels in neurons, with subsequent inhibition of neurotransmitter release (Di Marzo et al. 2004).

So far, few data are available with regards to the distribution of G-protein receptor 55 (GPR55). The expression of this receptor has been shown by fluorescence *in situ* hybridization in the spleen and in some regions of the human brain, such as the caudate nucleus and putamen (Sawzdargo et al. 1999). An important presence of this receptor has also been demonstrated in the neurons of the human DRG (Lauckner et al. 2008). In rat brain, GPR55 mRNA was detected by *in situ* hybridization in hippocampus, thalamic nuclei and regions of the midbrain (Brown 2007) and in the

periphery GPR55 mRNA was present in the rat spleen (Sawzdargo et al. 1999). The GPR55 has distinct intracellular signalling responses in comparison with CB_1 and CB_2 receptors (Lauckner et al. 2008). Thus, the activation of this receptor induces the increase of intracellular calcium via Gq and phospholipase C, and the inhibition of potassium current through M-type potassium channels, which includes an increase in neuronal excitability (Lauckner et al. 2008).

ii. Endogenous and exogenous ligands

The endogenous ligands for cannabinoid receptors, the endocannabinoids, are long chain polyunsaturated fatty acids derived from the membrane phospholipids, specifically from the arachidonic acid, and belong to the family of the eicosanoid compounds. The first isolated endocannbinoid was the ethenolamide of arachidonic acid, anandamide (Devane et al. 1992). The *in vivo* release of anandamide and its involvement in pain control was soon demonstrated (Schmid et al. 2000). Anandamide is synthesised from the phosphatidylethanolamine present on the cell membrane by the subsequent action of two enzymes: the N-acyltransferase and phospholipase D (Di Marzo et al. 1994). After release, anandamide is transported from the synaptic cleft inside the cell through passive diffusion or by a selective transporter that can be selectively inhibited by different compounds, such as *N*-(4-hydroxyphenyl)-arachidonamide (AM404) (Fegley et al. 2004). However, this transporter has not been yet identified. At present, it is postulated that anandamide diffuse passively thought the membrane and is then cached in the cytoplasm by the Faty Acid Binding Protein (FABP) and transported to the mitocondria (Kaczocha et al. 2009) where the enzyme that catabolizes anandamide, FAAH, is located (Cravatt et al. 1996) (Figure 5).

The most abundant endocannabinoid in the brain is 2-AG (Mechoulam et al. 1995; Sugiura et al. 1995). High levels of 2-AG are found in the brain and its concentration is about 200 times higher than anandamide (Stella et al. 1997). 2-AG is generated from diacylglycerol that is synthesised from phosphoinositides or from phosphatidic acid (Bisogno et al. 2005). The synthesis of 2-AG is mediated mainly by the phospholipase C (Piomelli 2003). The 2-AG reuptake is taking place by similar mechanisms than for anandamide. 2-AG degradation is mainly due to the action of the MAGL (Dinh et al. 2002) (Figure 5).

Other endogenous cannabinoids that have been identified are the 2-arachidonylglycerol ether, also called noladin ether (Hanus et al. 2001), virodhamine that has been proposed as an endogenous antagonist of CB_1 receptor (Porter et al. 2002) and N-arachidonoyldopamine (NADA), a vanilloid agonist with CB_1 affinity (Huang et al. 2002). Other two endogenous compounds with cannabinomimethic actions, but without affinity for the cannabinoid receptors, are oleylethanolamide (OEA) and palmythoilethanolamine (PEA) (Lambert and Di Marzo 1999). OEA at high concentrations is able to displace other ligands from the cannabinoid receptors (Lambert and Di Marzo 1999), and can reduce food intake from a peripheral mechanism (Rodriguez De Fonseca et al. 2001). PEA exerts antiinflammatory actions blocked by CB_2 antagonists (Calignano et al. 1998; Calignano et al. 2001; Conti et al. 2002), has antiepileptic properties and inhibits the intestinal motility in mice (Lambert et al. 2001).

Among the exogenous ligands, more than 60 cannabinoids have been found in *Cannabis Sativa*. The most important are the Δ9-THC, cannabinol and cannabidiol (CBD).

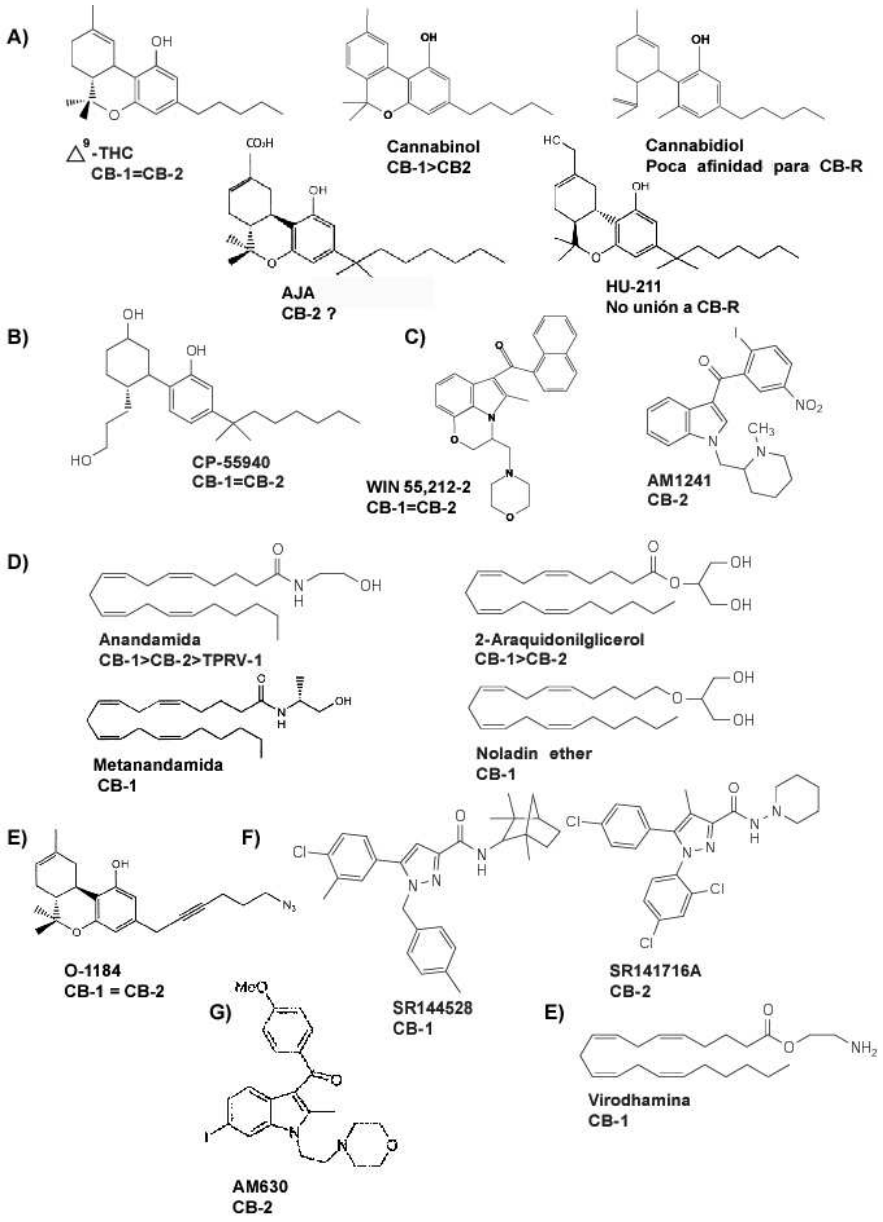

Figure 6. Cannabinoid ligands. Classical cannabinoid agonists (A), non-classical (B), aminoalkylindoles (C) and eicosanoids (D). Classical cannabinoid antagonists (E), diarylpirazoles (F), aminoalkylindoles (G) and eicosanoids (E). **CB-R:** cannabinoid receptors; **?:** no binding to CB_2, but similar effects; **TPRV-1:**vanilloid receptor. (Nadal and Baños, 2005)

Δ9-THC acts as a partial agonist at both CB_1 and CB_2 receptors and is the major responsible of the psychoactive effects of *Cannabis Sativa*. The psychoactive effects are attributed to the action on CB_1 (Zimmer et al. 1999; Huestis et al. 2001) and the immunomodulatory effects to the action on CB_2 (Kaminski 1998; Klein et al. 1998; Buckley et al. 2000). Cannabinol have only one tenth of the psychoactive activity of Δ9-THC. CBD is not psychoactive but can decrease the psychoactive effects of Δ9-THC (Zuardi et al. 1982), and have immunosupressive, antiinflamatory (Srivastava et al. 1998; Malfait et al. 2000), neuroprotective (Hampson et al. 1998) and antipsychotic activities (Zuardi et al. 2006 for a Review). From the natural cannabinoids two analogs with clinical application were synthesized: dronabinol ((Levo)-Δ9-THC) (Marinol, Roxana Laboratories) and nabilone (Cesamet, Eli Lilly).

Studies that link the structure of natural cannabinoids with their pharmacological activity and the cloning of cannabinoid receptors (Matsuda et al. 1990; Munro et al. 1993) has allowed the development of new molecules that selectively bind to the cannabinoid receptors. The figure 6 described the structure and affinity for the cannabinoid receptors of the principal cannabinoids classified by action and chemical structure. Nowadays, the synthetic agonists of the cannabinoid receptors can be classified into two main categories: (1) classical cannabinoids: compounds that have similar structure to THC, such as HU-210, CP-55,940 and nabilone; and (2) non classical cannabinoids: compounds with different chemical structure, such as the aminoalkylindoles, that include WIN 55,212-2.

The synthetic compounds have different intrinsic activity and affinity for the cannabinoid receptors (Howlett 2002). Thus, CP-55,940 and WIN

55,212-2 show a higher affinity for CB_1 and CB_2 receptors than THC. In addition, CP-55,940 has the same affinity for both CB_1 and CB_2, while the affinity of WIN 55,212-2 is slightly higher for CB_2 (Pertwee 2008).

Studies about the relationship between structure and activity have allowed the development of selective cannabinoid receptor antagonists. Thus, SR141716A (rimonabant) (Rinaldi-Carmona et al. 1994) and SR144528 (Rinaldi-Carmona et al. 1998) were the first selective antagonists for CB_1 and CB_2 receptors, respectively.

c. Cannabinoid system and pain modulation

The endocannabinoid system plays an important role in multiple aspects of the neural functions including learning and memory, emotion, addictive-like behaviour, feeding and metabolism, pain and neuroprotection. It is also involved in the modulation of different processes at the cardiovascular and immunological levels, among others. The distribution of the CB_1 receptors in the brain correlates with the pharmacological actions of the cannabinoids. Its high density in the basal ganglia is associated with the pronounced effects on the locomotor activity. The presence of the receptor in the hippocampus and cortex are related with the effects in learning and memory, and with the psychotropic and antiepileptic properties. The low toxicity and lethality are related with the low expression of receptors in the brainstem (Berrendero 2002). The endocannabinoid system interacts with multiple neurotransmitters, such as acetylcholine, dopamine, GABA, histamine, serotonin, glutamate, norepinephrine, prostaglandins and opioid peptides (Dewey 1986; Grotenhermen 2004). The interaction with these neurotransmitters is

responsible of most of the pharmacological effects of cannabinoids (Grotenhermen 2004).

The endocannabinoid system modulates the antinociceptive responses by acting at several central and peripheral levels. At the peripheral level, the activation of both CB_1 and CB_2 receptors participates in the antinociceptive effects of cannabinoids. In this line, several studies have proposed the existence of a synergism between the responses mediated by CB_1 and CB_2 receptors in the periphery (Malan et al. 2001). At this level, CB_1 and CB_2 activation have different mechanism of action. CB_1 action is mainly expressed at nerve terminals and is basically involved in the control of the neuron responses. In contrast, immune cells and keratinocytes seem responsible of the peripheral CB_2 analgesic action, where CB_2 receptors reduce the release of pronociceptive molecules from these cells (Malan et al. 2002; Ibrahim et al. 2005).

At the CNS, the antinociceptive effects induce by cannabinoids are mainly due to CB_1 located in the spinal cord and supraspinal structures (Meng et al. 1998; Ledent et al. 1999). At the spinal level, the CB_1 receptors are found mainly in the dorsal horn. Most of the primary afferent neurons that express CB_1 mRNA are Aβ fibers or large diameter fibers, involved in the sensitive, non-nociceptive transmission (Hohmann and Herkenham 1999). However, CB_1 receptors are also expressed in nociceptive fibers with small diameter including C fibers, and may inhibit the release of neurotransmitters involved in pain transmission (Drew et al. 2000; Kelly and Chapman 2001; Wilson and Nicoll 2001) (Figure 7). CB_1 mRNA is also highly expressed in the DRG (Hohmann 2002; Bridges et al. 2003). At this level, stimulation of CB_1 receptors blocks the presynaptic Ca^{2+} dependent channels, decreasing by this mechanism the release of neurotransmitters involved in pain transmision (Millns et al. 2001). In

addition, it has been recently shown that CB_2 receptors also participate in pain modulation in the spinal cord (Taylor 2009).

At the supraspinal level, cannabinoids are also able to modify the subjective interpretation of pain by modulating the neuronal activity mainly at the level of the limbic structures, such as amygdale (Manning et al. 2003). Cannabinoids may also inhibit pain transmission in supraspinal structures acting at the level of the thalamus (Martin et al. 1996; Martin et al. 1999a).

Endocannabinoid system plays an outstanding role in the control of the ascending pathways involved in the transmission of nociceptive stimuli. However, another central mechanism for cannabinoid antinociceptive effects is the modulation of the descending inhibitory pathways. Several studies demonstrated the presence of a bidirectional control of pain transmission in the PAG matter and RVM that can exert both inhibitory and facilitatory control (Fields 2004). Microinjection of cannabinoid agonists into the PAG (Martin and Lichtman 1998; Martin et al. 1999a) and RVM (Martin et al. 1998), as well as the electrostimulation of these areas (Fields et al. 1991), resulted in analgesia by enhancing the activity of "off cells" by a cannabinoid dependent mechanism. The cannabinoids may stimulate the descending inhibitory pathway by activating neurons from both brain regions. This activation seems to be induced by the inhibition of GABA release in the axon terminal of presynaptic interneurons located in RVM and PAG through a mechanism similar to that described for opioid analgeisa (Vaughan et al. 2000; Rea et al. 2007) (see chapter 4. C.).

The analgesic effects of cannabinoid compounds have been demonstrated in multiple behavioural studies in both animals and humans (Martin and

Lichtman 1998; Pacher et al. 2006). The most employed among acute nociceptive models that apply thermal stimuli to reveal cannabinoid antinociception are the hot plate (Buxbaum 1972; Martin 1985; Hutcheson et al. 1998) and tail flick paradigms (Buxbaum 1972). These two tests evaluate different behavioural responses. While the antinociceptive responses obtained in the hot plate test are mainly modulated by the activation of supraspinal pathways, the behavioural responses observed in the tail flick paradigm are mainly due to spinal mechanisms. Cannabinoids agonists proved to be efficient in both tests (Lichtman and Martin 1991b; Martin and Lichtman 1998; Hohmann 2002; Romero et al. 2002; Tham et al. 2005). Cannabinoid agonists induced also antinociceptive effects in mechanical models that measure motor (Smith et al. 1994) or reflex (Gilbert 1981) responses, chemical models such as the writhing response induced by acetic acid or the administration of fenilbenzoquinone (Welch et al. 1995; Ulugol et al. 2006) and models of electric stimulation of paw (Weissman et al. 1982), sciatic nerve (Bicher and Mechoulam 1968) or dental pulp (Kaymakcalan et al. 1974). The cannabinoid agonists also produced antinociceptive effects in models of inflammatory pain such as the hyperalgesia induced by carrageenan (Mazzari et al. 1996), capsaicin (Li et al. 1999), formalin (Moss and Johnson 1980; Calignano et al. 1998) and Freund's adjuvant (Martin et al. 1999b).

In humans, cannabinoid agonists are already used to alleviate some manifestations of pain. Indeed, Sativex® (Δ9-THC with CBD) is prescribed for the symptomatic relief of neuropathic pain in adults with multiple sclerosis and as an adjunctive treatment for adult patients with advanced cancer (Pertwee 2009). One important challenge would be to identify additional therapeutic targets for cannabinoid agonists considering the promising findings already reported.

d. Cannabinoid drugs and neuropathic pain

One of the ancestral uses of cannabis was to treat pain. Historical documents reveal the use of cannabis for surgical anaesthesia in ancient China and for relieving pain of diverse origin in ancient Israel, Greece, Rome, and India (Mechoulam and Hanus 2000). Cannabinoids exert their antinociceptive effects through complex mechanisms involving effects on peripheral sensory nerves (Calignano et al. 1998; Fox et al. 2001; Johanek and Simone 2004; Jordt et al. 2004; Amaya et al. 2006), spinal cord (Lichtman and Martin 1991a; b; Chapman 1999; Drew et al. 2000; Naderi et al. 2005; Suplita et al. 2006) and brain (Martin et al. 1993; Meng et al. 1998; Hohmann et al. 1999; Fox et al. 2001). This is consistent with the anatomical location of CB_1 receptors in areas relevant to pain in the peripheral afferent neurons, DRG, spinal dorsal horn, and brain (Hohmann et al. 1999; Sanudo-Pena et al. 1999).

Recent studies have shown that the cannabinoids were also effective in models of neuropathic pain (Pertwee 2001; Goya et al. 2003; Jhaveri et al. 2007; Rahn and Hohmann 2009). Thus, an up-regulation of spinal CB_1 receptor was revealed after a chronic constriction of sciatic nerve that promotes the enhancement of the analgesics effects of Win 55,212-2 on neuropathic pain in rats (Lim et al. 2003). By contrast, a genetic study using CB_1 KO mice, has shown that CB_1 cannabinoid receptors are not critically involved in the development of neuropathic pain nor in the anti-allodynic and anti-hyperalgesic effects of gabapentin in a model of neuropathic pain induced by partial sciatic nerve ligation (Castane et al. 2006). In spite of these results, the peripheral CB_1 receptors are involved in neuropathic pain states as it was revealed using tissue specific KO mice

lacking the CB_1 selectively in the Nav1.8$^+$ expressing peripheral neurones (Agarwal et al. 2007) (Figure 7).

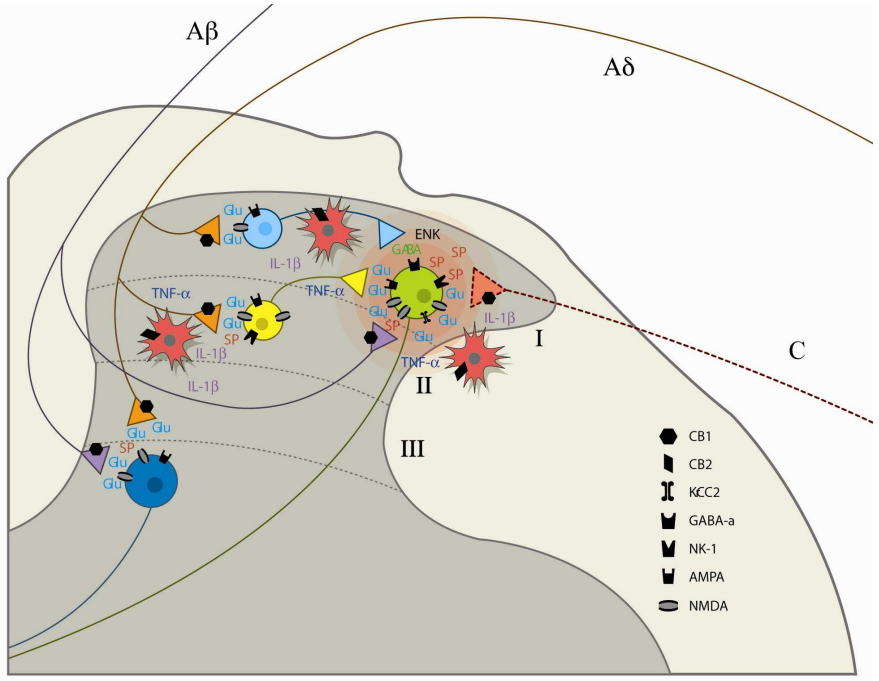

Figure 7. Implication of endocannabinoid system at the spinal level in the pathophysiology of neuropathic pain. CB_1 receptor is mainly expressed presynaptic at the tactile afferent Aβ fibers. It is present in less extend in the axon buttons of the Aδ and C fibers. As explained in the figure 2, the Nav1.8$^+$ channel is up regulated in the injured C and Aδ fibers, the CB_1 receptors located on this fibers are crucial for the cannabinoid analgesia. The presence of presynaptic CB_1 in the terminals of C, Aδ and Aβ fibers can inhibit or reduce the release of glutamate and substance P from the afferent injured terminals reducing the excitatory input that generates the C fibers on the nociceptive neurons (green), or reducing the activation of the glutamatergic/substance P excitatory interneurons (yellow) caused by Aδ fibers. This cannabinoid response indirectly reduces the excitatory input of the nociceptive neurons (green). The CB_2 receptor is expresed in the injured spinal cord and is located in the activated microglia cells. CB_2 receptor stimulation reduces the activation of the microglia and the inhibition of the release of proinflammatory cytokines as TNF-α and IL-1β that produce neuronal sensitization. CB_1 and CB_2 actions are able to reduce the manifestations of neuropathic pain, such as hyperalgesia and allodynia.

The cannabinoids are analgesic agents with strong evidence of efficacy in animal models of neuropathic pain and increasing evidence of efficacy in humans. In clinical trials, dronabinol has been shown to produce modest analgesia in a randomized trial of central pain in multiple sclerosis patients (Svendsen et al. 2004). Sativex® provided significant benefit in another trial of central pain in multiple sclerosis (Rog et al. 2005). At the present moment, Sativex® is approved in the UK, Spain, Czech Republic, Canada and New Zealand, as a treatment of multiple sclerosis spasticity. In addition, clinical trials are ongoing, some of them with initial promising results to evaluate the effectiveness of Sativex® in the treatment of cancer pain, peripheral neuropathic pain (Nurmikko et al. 2007) and in the sleep disorders associated with chronic pain (Russo et al. 2007). Moreover, the *Cannabis sativa* female fower tops are prescribed as a treatment for some illnesses in Canada, Netherlands, Israel, Germany and some states of U.S.A.

e. CB_2 receptors as a new therapeutic target

CB_2 cannabinoid receptors have also been involved in the development of neuropathic pain. In the spinal cord, CB_2 receptor expression, in parallel with the simultaneous enhanced expression of activated microglia, was induced during neuropathic pain produced by peripheral nerve injury (Zhang et al. 2003).

Microglial cells seem to be involved in the development of neuropathic pain through the release of several cytokines, which are known to produce neural sensitization in the spinal cord (DeLeo and Yezierski 2001; Clark et al. 2007) (Figure 7). Interestingly, CB_2 receptor stimulation has been reported to attenuate microglial activation (Ehrhart et al. 2005; Romero-

Sandoval and Eisenach 2007) and CB_2 agonists induce analgesic effects in neuropathic pain models (Ibrahim et al. 2003). Although these studies suggest a potential role for CB_2 receptors in the modulation of neuropathic pain, the mechanisms underlying these analgesic effects and the exact involvement of CB_2 receptors in the development of neuropathic pain have not been clarified yet.

6. Neuroimmune interactions and neuropathic pain

a. General aspects

The previous chapters were devoted to the pathophysiology of the neuropathic pain with special focus in the neuronal mechanisms. However, the immunological component, which interacts directly with the neuronal elements, also plays an important role in the biological substrate of neuropathic pain. These two components sinergically interact to develop and maintain the neuropathic pain and their associated symptoms. Some models of neuropathic pain are caused by an induction of neuroinflammation via injection of Freund's adjuvant, TNF and NGF (Wagner and Myers 1996; Eliav et al. 1999; Ruiz et al. 2004). In addition, a reactive gliosis that affects microglia and astrocytes is generated in all animal models of neuropathic pain (Colburn and DeLeo 1999). Therefore, the neuroimmune alterations that occur during neuropathic pain represents a key component of this pathological state.

b. Peripheral mechanisms

The main peripheral immunological component in the neuropathic pain is the macrophage infiltration in the site of injury in the nerve and in the DRG. This infiltration leads to a neuroinflamation in the injured nerve and the DRG leading to an activation of microglia and satellite cells (astrocytes). An aggregation of satellite cells surrounding degenerating sensory neuron soma occurs in peripheral ganglia under these pathological conditions (Thomas et al. 1992) (Figure 8). Recent data demonstrate that

DRG satellite cells and macrophages undergo morphological changes (Woodham et al. 1989; Stephenson and Byers 1995) and up-regulate a variety of cytokines and growth factors following spinal or sciatic nerve ligation (Taniuchi et al. 1986; Lindholm et al. 1987; Meyer et al. 1992; Hammarberg et al. 1996). Thus, peripheral sensitization of adjacent uninjured sensory neurons by proinflammatory cytokines derived from satellite cells (Zhou et al. 1999) and/or infiltrating macrophages (McMahon et al. 2005) may contribute to the development of the neuropathy. This neuroinflamation in the DRG seem responsible for the release of the NGF that triggers the sprouting of sympathetic neurons (Sebert and Shooter 1993), which was explained in the neuropathic pain chapter (Chapter 2. C. i.) (Figure 8).

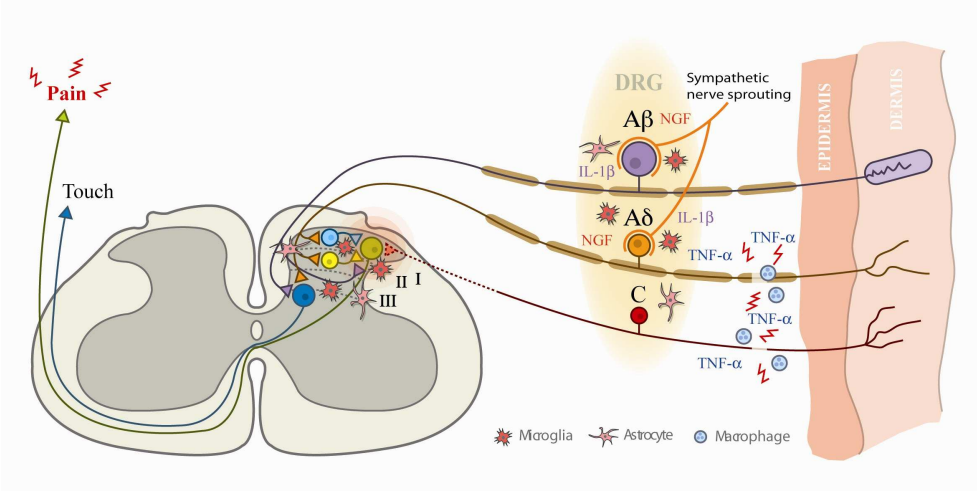

Figure 8. Immune responses in the periphery and spinal cord during neuropathic pain. An infiltration of macrophages occurs during neuropathic pain mainly in the site of nerve injury and in the DRG, that release TNF-α and IL-1β. This infiltration leads to the activation of the microglia and astrocytes in the DRG producing the release of IL-1β, NGF, among others. As it is explained in the figure 2, NGF produced by the microglia and astrocytes promotes the sympathetic sprouting. In the spinal cord, the degenerating axons of C fibers leads to the activation of the microglia. These cells release IL-1β that sensitizes the nociceptive neurons (green) and interneurons (yellow and blue), which leads to the activation of the astrocytes in the spinal cord completing the process of

neuroinflamation that produces the sprouting of Aβ fibers and chronifies the neuropathic pain.

c. Central mechanisms

Central sensitization occurs in the spinal cord by pathophysiological mechanisms that involve glial cells, mainly microglia and astrocytes. Spinal microglia activation has been observed in many experimental models of neuropathic pain (Watkins et al. 2001) (Figure 8), as shown by proliferation, cell recruitment at the site of the injury, and increased expression of immunomolecules (Colburn et al. 1997; Tsuda et al. 2005). Microglia and astrocytes are activated in several pathological conditions associated with enhanced pain (Wieseler-Frank et al. 2005). Thus, peripheral nerve injury activates spinal cord glia and these activated cells enhance pain by releasing glutamate (Wieseler-Frank et al. 2005) and neuroexcitatory glial proinflammatory cytokines TNF, interleukin 1β (IL-1β) and interleukin 6 (IL-6). The complete neuroinflammatory process leading to the progression of the neuropathic pain requires the coactivation of both microglia and astrocytes (Colburn and DeLeo 1999) (Figure 8 and 9). Cytokines that are released by the activated microglia seem responsible for the subsequent activation of astrocytes, which permits the consolidation of the neuropathic pain state (John et al. 2004). This role of microglia in neuropathic pain seems to be related with the release of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α (DeLeo and Yezierski 2001). The blockade of glial activation or the actions of glial-derived substances have been shown to be an efficient mechanism for preventing and reversing neuropathic pain (Sweitzer et al. 2001a; Sweitzer et al. 2001b). Microglia, but also astrocytes, release cytokines, which are known to produce neuronal sensitisation at the level of the spinal cord (Meller et al. 1994). Therefore, it is reasonable to suspect that glial activation after nerve injury may play an important role

in nociceptive processing, and also in synaptic remodelling that occurs in this pathological situation.

Some manifestations of neuropathic pain seem to precede microglial activation and some data have shown that both phenomena are not correlated (Colburn et al. 1997). However, activation is profound following peripheral nerve injury, and this relationship is clearly related in some experimental conditions to the manifestations of neuropathic pain in different experimental models (Colburn and DeLeo 1999) (Figure 8 and 9). Indeed, microglial proliferation in spinal cord increases with aging and it is still increased further after peripheral nerve injury, and these changes are correlated with the manifestations of neuropathic pain (Stuesse et al. 2000).

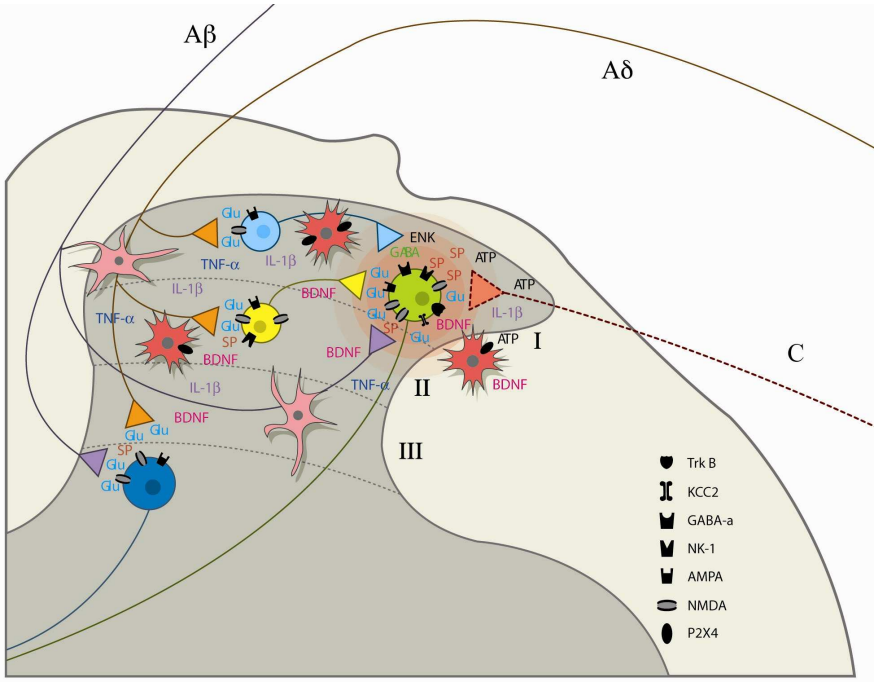

Figure 9. Neuroimmune interactions in the spinal cord during neuropathic pain. The process that leads to the development of neuropathic pain state is

initiated by the ATP released by the degenerating C fibers terminals. Its interaction on the P2X4 receptor of the microglial cells produces their activation. The activated microglia releases proinflamatory cytokines, such as TNF-α and IL-1β, that sensitize the nociceptive neurons (green) and interneurons (yellow and blue), and activates the astrocytes leading to the complete neuroinflammation. In addition, activated microglia releases neurothrophic factors such as BDNF that activates the Trk B receptor from the nociceptive neurons (green) which causes a down-regulation of KCC2. BDNF also changes the membrane potential, which produces excitation in the nociceptive neurons (green), when these neurons are stimulated by GABA or other inhibitory inputs. Moreover, BDNF seems to be the responsible of the sprouting of the Aβ fibers from the deeper laminas to innervate the nociceptive neurons (green) of the superficial lamina I.

In agreement with a direct relationship between microglial activation and neuropathic pain, the inhibition of gliosis by minocicline, an inhibitor of microglial activation, is correlated with a reduction in mechanical allodynia and hyperalgesia. However, when it is given on day 5 post-nerve injury, the drug is unable to attenuate existing hyperalgesia and allodynia, even when microglial activation is significantly reduced (Raghavendra et al. 2003).

d. Activation of neurons and glia in chronic pain

The molecular mechanisms underlaying the link between microglial cells and neuronal activation have been recently suggested (Coull et al. 2005).Thus, spinal microglia stimulated by adenosine triphosphate (ATP) contributes to tactile allodynia after nerve injury (Tsuda et al. 2003). ATP and adenosine are released from the injured terminal axons in the spinal cord, and stimulate the microglia through the purinergic ionotropic 4 receptors (P2X4) (Inoue et al. 2004) and adenosine type 2A receptor (A2A) (Bura et al. 2008). The activation of P2X4 receptors lead to the initial activation of the microglial cells, but for the sustained stimulation of microglia is necessary the activation of A2A receptor (Bura et al. 2008). Microglial cells expres ecto-50-nucleotidase and it can also

dephosphorilate ATP to adenosine (Sawynok et al. 1998). The activation of P2X4 receptor leads to an increase in phospho-p38 MAPK (Trang et al. 2009), wich is entirely restricted to microglia and is not related with astrocytes (Jin et al. 2003). The activation of phospho-p38 produces an enhancement of production and release of BDNF from microglia (Trang et al. 2009). This activation may play a role in the early induction of neuropathic pain and later may cooperate to regulate the subsequent development and consolidation of neuropathic pain. Brain derived neurotrophic factor (BNDF) is a crucial signalling molecule between microglia and neurons (Coull et al. 2005). Application of BNDF mimics the shift in the anion reversal potential of nociceptive neurons occurring after peripheral nerve injury (Coull et al. 2003) (Figure 9). Thus, the blockade of the main BDNF receptor, the tyrosine kinase B receptor (TrkB) reverses both allodynia and the shift that follows nerve injury and ATP-stimulated microglia. In turn, ATP evokes the release of BNDF from microglia. The prevention of BNDF release from microglia also inhibits the effects of these cells on the hyperalgesia and anion reversal potential (Coull et al. 2005).

At the nociceptive neurons, BDNF acts on Trk B receptors located in lamina I. This action induces the down regulation of KCC2, wich produces a depolarizing shift in the anion reversal potential. As a consequence of this effect, the polarity of currents activated by GABA is inverted (Figure 9). Therefore, neurons are depolarised rather than hyperpolarized after GABA receptor stimulation, wich contributes the to hyperexcitability and the manifestations of neuropathic pain due to these plasticity changes and the subsequent excitotoxicity gated by the depolarising anion currents (Coull et al. 2005).

In addition, the release of BDNF from the microglial cells seems to be the responsible of the sprouting of dopamine fibers in the striatum caused by injury (Batchelor et al. 1999). Although the neuropathic pain state differs from striatal injury, the role of the BDNF released by the activated microglia can be similar in the two states. We can hypothesize that BDNF gradient created in the neuroinflamed spinal cord acts as the neurotrophic and quimiotactic factor responsible of the sprouting of the Aβ fibers from the inner laminas to the affected superficial lamina I and II. This change would be able to generate the allodynia and hyperalgesia commonly produced during neuropathic pain (Figure 9).

OBJECTIVES

- To investigate the involvement of the endogenous opioid system in the development and expression of neuropathic pain.

- To explore the participation of DOR of nociceptive sensory neurons in the development and expression of chronic pain.

- To analyze the involvement of the endocannabinoid system through CB_2 receptors in the development and expression of neuropathic pain.

- To examine the involvement of interferon-gamma (IFN-γ) in the CB_2 mediated changes in the development and expression of neuropathic pain.

MATERIAL AND METHODS / RESULTS

. Chapter 1 Neuropathic pain is enhanced in delta-opioid receptor knockout mice. Nadal X, Baños JE, Kieffer BL, Maldonado R. Eur J Neurosci. 2006 Feb;23(3):830-4.

Objectives

To evaluate the possible involvement of DOR in the development and expression of neuropathic pain.

Material and Methods

We use constitutive KO mice and wild-type littermates for DOR that were exposed to neuropathic pain by partial sciatic nerve ligation (PSNL). The plantar test was used for the assesment of hyperalgesia and the von Frey model and the cold plate test for the evaluation of allodynia.

Results

In wild-type and DOR knockout mice, sciatic nerve injury led to a neuropathic pain syndrome revealed in the three nociceptive behavioural tests. However, the development of mechanical and thermal allodynia, and thermal hyperalgesia was significantly enhanced in DOR knockout mice.

Conclussions

These results reveal the involvement of DOR in the development of neuropathic pain and suggest a new potential therapeutic use of DOR agonists.

Nadal X, Banos JE, Kieffer BL, Maldonado R. Neuropathic pain is enhanced in delta-opioid receptor knockout mice. Eur J Neurosci. 2006 Feb;23(3):830-4

. Chapter 2 Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. Gaveriaux-Ruff C, Nozaki C*, Nadal X*, Hever XC*, Weibel R, Matifas A, Reiss D, Filliol D, Nassar MA, Wood JN, Maldonado R, Kieffer BL. Pain. 2011 Feb 2. [Epub ahead of print]
*Equally contributed

Objectives

- To evaluate the contribution of peripheral DOR in acute pain control.
- To evaluate the possible involvement of peripheral DOR in the development and expression of inflammatory and neuropathic pain.
- To evaluate the involvement of peripheral DOR in the analgesic effects of opioid compounds.

Material and Methods

We generated a conditional KO mouse where DOR are deleted specifically in peripheral Nav1.8-positive primary nociceptive neurons. In these animals, acute nociceptive responses were evaluated by using the plantar test for the assesment of hyperalgesia and the von Frey model and the cold plate test for the evaluation of allodynia. Inflammatory pain was induced by the administration of complete Freund's adjuvant (CFA) and neuropathic pain by partial sciatic nerve ligation (PSNL).

Results

Mutant mice showed normal pain responses to acute heat, mechanical and formalin stimuli. Mutant animals showed a remarkable increase of mechanical allodynia induced by both inflammatory (CFA) and neuropathic pain conditions (PSNL). The delta agonist SNC80, administered systemically (CFA and PSNL models) or intra-paw (PSNL),

dose-dependently reduced thermal hyperalgesia and mechanical allodynia in control mice. SNC80 analgesic effects were absent in conditional mutant mice.

Conclussions

These results reveal the existence of DOR-mediated mechanisms, which operate at the level of Nav1.8-positive nociceptive neurons in the control of pain. DOR in these neurons tonically inhibit mechanical hypersensitivity in both inflammatory and neuropathic pain, and are essential to mediate DOR analgesia under conditions of persistent pain.

Gaveriaux-Ruff C, Nozaki C, Nadal X, Hever XC, Weibel R, Matifas A, et al. Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. Pain. 2011 Jun;152(6):1238-1248.

. Chapter 3 Crucial role of CB (2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain. Racz I*, Nadal X*, Alferink J*, Baños JE, Rehnelt J, Martín M, Pintado B, Gutiérrez-Adan A, Sanguino E, Manzanares J, Zimmer A, Maldonado R. J Neurosci. 2008 Nov 12;28(46):12125-35.
*Equally contributed

Objectives

To clarify the role played by CB_2 cannabinoid receptors in the regulation of the central immune responses leading to the development of neuropathic pain.

Material and Methods

We use constitutive CB_2 KO mice, transgenic mice overexpressing CB_2 receptors at CNS level and irradiated wild-type mice reconstituted with bone marrow cells from CB_2 KO in a model of partial sciatic nerve ligation (PSNL). The plantar test was used for the assessment of hyperalgesia and the von Frey model and the cold plate test to evaluate allodynia. The glial changes in the spinal cord were evaluated with immunohystochemistry staining of Iba-1 (microglial cells) and GFAP (reactive astrocytes).

Results

CB_2 KO mice and wild-type littermates were exposed to sciatic nerve injury. Both genotypes developed a similar hyperalgesia and allodynia in the ipsilateral paw. However, CB_2 KO also developed a contralateral mirror-image pain, associated with an enhanced microglial and astrocytic expression in the contralateral spinal horn. In transgenic mice overexpressing CB_2 receptors the hyperalgesia, allodynia, microglial and

astrocytic activation induced by sciatic nerve injury were attenuated. The enhanced manifestations of neuropathic pain were replicated in irradiated wild-type mice reconstituted with bone marrow cells from CB_2 KO.

Conclusions

These results demonstrate the crucial role of CB_2 cannabinoid receptor in modulating glial activation in response to nerve injury, and demonstrate the implication of the CB_2 receptor expressed in hematopoietic cells in the development of neuropathic pain.

Racz I*, Nadal X*, Alferink J*, Banos JE, Rehnelt J, Martin M, Pintado B, Gutierrez-Adan A,Sanguino E, Manzanares J, Zimmer A, Maldonado R. Crucial role of CB (2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain. J Neurosci. 2008 Nov 12;28(46):12125-35.

. Chapter 4 Interferon-gamma is a critical modulator of CB (2) cannabinoid receptor signalling during neuropathic pain. Racz I*, Nadal X*, Alferink J*, Baños JE, Rehnelt J, Martín M, Pintado B, Gutiérrez-Adan A, Sanguino E, Bellora N, Manzanares J, Zimmer A, Maldonado R. J Neurosci. 2008 Nov 12;28(46):12136-45.
*Equally contributed

Objectives

To investigate the mechanisms leading to the enhanced manifestation of neuropathic pain due to the absence of CB_2 receptors.

Material and Methods

We use the same animals than in the third chapter of the Material and Methods/Results plus the interferon-γ (IFN-γ) KO and the double IFN-γ/CB_2 KO mice in the PSNL model of neuropathic pain. For the assessment of hyperalgesia and allodynia we use the same test than in the third chapter. We use microarray analysis to establishing expression profiles of spinal cord tissues, and observe the glial changes in the spinal cord with immunohystochemistry staining of Iba-1 (microglial cells), GFAP (reactive astrocytes) and IFN-γ. In addition we use cell culture of BV-2 microglial cells to reveal transcriptional changes induced by IFN-γ.

Results

Microarray analysis revealed an enhanced IFN-γ response in the absence of CB_2 signalling by determination of the expression profiles of spinal cord tissues from wild-type and CB_2 deficient mice after nerve injury.
Immunofluorescence staining demonstrated an IFN-γ production by astrocytes and neurons ispilateral to the nerve injury in wild-type animals.

CB_2 deficient mice showed neuronal and astrocytic IFN-γ immunoreactivity both in the ipsilateral and the contralateral region, thus matching the pattern of nociceptive hypersensitivity in these animals. Experiments in BV-2 microglia cells revealed that transcriptional changes induced by IFN-γ in two key elements for neuropathic pain development, iNOS and CCR2, are modulated by CB_2 receptor signalling. Using a double KO mouse strain deficient in CB_2 receptors and IFN-γ, we obtained direct support for a functional involvement of IFN-γ as a mediator of CB_2 signalling. These double mutants did not show the enhanced manifestations of neuropathic pain observed in CB_2 KO.

Conclusions

These data clearly demonstrate that the CB_2 receptor mediated control of neuropathic pain is IFN-γ dependent.

IFN-γ promotes microglia activation by the induction of several inflammatory pathways**,** including an enhancement in iNOS and CCR2 activity. The activity of CB_2 receptors in microglial cells would reduce the activation of these cells during neuropathic pain by regulating the expression of iNOS and CCR2.

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DISCUSSION

Role of DOR in neuropathic pain

The enhanced behavioural manifestations of neuropathic pain in DOR KO mice indicate the participation of these receptors in the development of neuropathic pain. A significant enhancement of thermal hyperalgesia, mechanical allodynia and thermal allodynia was observed in constitutive DOR KO mice exposed to a partial sciatic nerve ligation. This pain enhancement in DOR KO mice could be explained by the existence of an endogenous opioid tone under neuropathic pain conditions that acts on DOR, which would attenuate the severity of the pain manifestations under these pathological conditions.

Previous studies using DOR and combinatory KO mice have revealed that the endogenous opioid tone on DOR does not seem to play an important role in the control of acute nociceptive responses (Martin et al. 2003). However, such an endogenous DOR tone modulates the nociceptive inflammatory responses induced by formalin (Martin et al. 2003). In agreement, the administration of DOR antagonists and the inhibition of leu-enkephalin activity by specific antisera increase the nociceptive responses induced by formalin in rats (Ossipov et al. 1996). These previous studies and our present findings indicate the possible existence of a tonic activation of DOR during inflammatory and neuropathic pain that could counteract the manifestations of these processes. Our analysis of mice lacking DOR in Nav1.8^{+} primary afferent neurons, and the comparison with data from constitutive DOR KO mice and from pharmacological studies, confirm that DOR are weakly involved in acute pain perception. In contrast, this particular population of peripheral DOR tonically inhibits mechanical allodynia under conditions of both

inflammatory and neuropathic pain, and their presence is mandatory for delta agonist-induced analgesia in these models of chronic pain.

DOR are expressed in all categories of sensory neurons including nociceptive and non-nociceptive neurons, as well as in the spinal cord and higher-order nociceptive networks in the brain (Mansour et al. 1995; Wang and Wessendorf 2001). A reduction of DOR immunoreactivity was observed at superficial lamina of the dorsal horn during neuropathic pain (Stone et al. 2004). MOR and KOR density in lumbar spinal cord did not change after spinal nerve ligation (Wang et al. 2001). However, other study did not found changes in DOR mRNA and found a decrease of MOR mRNA in the ipsilateral dorsal root ganglion and lumbar spinal cord (Pol et al. 2006). Activation of presynaptic DOR in C fibers produces neuron hyperpolarization and inhibits the excitatory glutamatergic synaptic transmission in the spinal dorsal horn (Glaum et al. 1994), inhibiting the ascending noxious stimuli. Other adaptive changes have been reported in the opioid system during neuropathic pain, including an enhancement in the spinal dynorphin content (Wang et al. 2001). Such a dynorphin enhancement seems to be essential in the maintenance of neuropathic pain (Wang et al. 2001; Xu et al. 2004). A high concentration of dynorphin would increase NMDA receptor activity producing pronociceptive effects and contributing to the sensitization of the spinal cord (Wang et al. 2001). DOR activity could reduce the dynorphin sensitization of the spinal cord during neuropathic pain by inhibiting the glutamatergic synaptic transmission. In addition, DOR was involved in the tonic spinal regulation of substance P levels by inhibiting its release after mechanical or thermal stimuli (Zachariou and Goldstein 1996).

Neuronal compensatory changes in these KO mice could have minimized the contribution of the DOR endogenous tone in the manifestations of

neuropathic pain. Noticeably however, compensatory modifications could not be evidenced in constitutive DOR KO mice at least within the other components of the opioid system, such as endogenous opioid peptides and other opioid receptors (Kieffer 1999).

In the conditional mutant animals, we have deleted DOR in small and medium diameter Nav1.8^{+} DRG neurons that represent the main population of primary nociceptive neurons, including unmyelinated C fibers and thinly myelinated Aδ fibers, while intact receptor expression was maintained in large diameter Aδ/Aβ somatosensory neurons. Our targeting strategy, therefore, successfully eliminated receptors that are potentially involved in pain control at the level of primary nociceptive processing. Our data demonstrate that these receptors play a critical role in the tonic inhibition of mechanical allodynia and in DOR analgesia. At this stage, the role of DOR expressed in large DRG neurons remains to be clarified. These large DRG neurons seem to be involved in the spinal reorganisation in response to peripheral nerve injury (Woolf and Mannion 1999). The Aβ fibers sprout into lamina II of the dorsal horn, which is normally innervated by C fibers. At this level, Aβ-fibers establish functional synaptic contact with other second order neurons that are involved in pain transmission (Scholz and Woolf, 2002). As a consequence of these synapses, low threshold non-noxious inputs from the Aβ-fibers can be interpreted as nociceptive in origin although they are not (Bridges *et al.,* 2001). Furthermore, Aβ fibers suffer a phenotypic switch and begin to express nociceptors as TRPV1 (Ma *et al.,* 2005), substance P and CGRP that have an excitatory effect on postsynaptic neurons. Thus, partial nerve injury and streptozotocin-induced diabetes produce a down-regulation of TRPV1 on many damaged afferent neurons and novel expression of TRPV1 on uninjured C fibers and A fibers (myelinated Aβ and Aδ) (Hong and Wiley, 2006; Hudson *et al.,* 2001). All these

nociceptors and neurokinins are normally expressed by primary afferent C fibers and Aδ fibers, but not in Aβ fibers (Miki *et al.,* 1998). After central sensitization, normally innocuous tactile stimuli become able of activating spinal cord pain-signalling neurons via Aδ and Aβ low-threshold mechanoreceptors (Tal and Bennett, 1994). Therefore the novel expression of TPRV1 channels in Aβ fibers can contribute to the thermal hyperalgesia.

DOR conditional mice show increased mechanic allodynic responses in animals exposed to both inflammatory and neuropathic pain, suggesting that a peripheral DOR-mediated tone normally inhibits chronic mechanical hypersensitivity. Mechanisms underlying this DOR activity likely involve endogenous opioid peptides, whose implication in decreasing pain perception has been shown previously (Zollner et al. 2008). Recently, it was shown that endogenous opioid peptides released from infiltrating immune cells at peripheral injury sites protect against neuropathic pain (Labuz et al. 2009). Together, these data and our findings suggest that DOR expressed by Nav1.8^{+} cells may respond to the release of injury-induced endogenous opioids.

Interestingly, mechanical allodynia but not thermal hyperalgesia, was increased in conditional mutant mice in both complete Freund's adjuvant (CFA) and PSNL models. This observation suggests that the endogenous DOR tone operating at the level of Nav1.8^{+} neurons influences mechanical rather than thermal nociceptive processing. A previous study demonstrated the preferential localization of DOR in IB4 positive DRG neurons, which mediate mostly mechanical nociception, and decreased mechanical allodynia, but not heat hyperalgesia upon intrathecal SNC80 administration (Scherrer et al. 2009). Hence, our present data on endogenous delta tone, and this previous study (Scherrer et al. 2009)

concur to show a preferential role of delta receptors in controlling mechanical sensitivity. This does not exclude the possibility that DOR expressed by IB4 positive cells (Wang et al.) may also control nociceptive inputs.

DOR conditional mice do not respond to systemic delta agonist administration, and this was observed in the two chronic pain models here investigated. This result demonstrates that delta receptors expressed in Nav1.8^{+} neurons are needed for systemic delta opioid analgesia. The genetic deletion in conditional mice leaves large populations of delta receptors intact, including receptors in other types of sensory DRG neurons or receptors expressed in nociceptive circuits at the CNS. Based on our data, these intact receptors do not seem capable of generating a detectable analgesic response after the systemic administration of the DOR agonist, at least in the absence of Nav1.8-DOR. However the activation of these receptors by the systemic administration of SNC80 seems responsible for the analgesic effects on the thermal hyperalgesia observed in wild-type mice. Previous studies have suggested that DOR potentially produce analgesia at different sites within the nociceptive circuitry. Accordingly, SNC80 or related compounds produce analgesia when injected into the paw (Stein et al. 1989; Pacheco and Duarte 2005; Kabli and Cahill 2007; Obara et al. 2009), but also by i.t. (Hosohata et al. 2000; Mika et al. 2001; Kawaraguchi et al. 2004; Guan et al. 2005; Gendron et al. 2007a; Scherrer et al. 2009), i.c.v. (Fraser et al. 2000; Hosohata et al. 2000) and intra-RVM (Hurley and Hammond 2000; Marinelli et al. 2005). We have shown here that DOR expressed by primary afferent Nav1.8^{+} cells are necessary for the analgesic efficacy of systemic and intra-paw administration of SNC80. These receptors, however, could not be sufficient to produce the full analgesic response of the DOR agonist and we cannot exclude a contribution of central

receptors at higher pain processing levels. Future studies using different driver Cre lines will evaluate the implication of other DOR populations in opioid analgesia.

Our present data show that SNC80 reduces in wild-type animals both mechanical allodynia and thermal hyperalgesia in CFA and PSNL models. This finding appears to disagree with our above conclusion that endogenous DOR activation inhibits preferably mechanical hypersensitivity. Taken into account the previous comments about the transmission of the thermal hyperalgesia by the sprouted Aβ fibers, this action of SNC80 could be better explained. It is likely that DOR activation after systemic SNC80 administration largely exceeds levels of endogenous activation, thereby extending analgesic efficacy of the drug to the heat response. DOR may preferentially control mechanical sensitivity, but also influence thermal sensitivity although to a lower extent. Hence, modulation of heat hypersensitivity may be less detectable, depending on the experimental design (i.e., endogenous or exogenous receptor activation, dosing, and the mode of drug administration). Noteworthy, several teams showed analgesic efficacy of systemic SNC80 or other delta agonists in reducing both mechanical or heat hypersensitivities (Table 4), which is consistent with the present pharmacological data. Importantly, we have shown here that this mechanical/thermal control occurs at the level of DOR in Nav1.8^{+} primary nociceptors. SNC80 analgesia is abolished in conditional mutants in two chronic pain paradigms (inflammation and neuropathy), but not in the early inflammatory formalin response. Also, our analysis of basal nociceptive responses reveals no phenotype in the conditional mice. The targeted peripheral receptors, therefore, are not involved in acute pain perception, but are critical to modulate chronic pain. This is in accordance with previous adaptations, including neuronal and glial changes that together contribute

to consolidate persistent pain (Julius and Basbaum 2001; Scholz and Woolf 2002; Ueda 2006; Holdridge and Cahill 2007; Watkins et al. 2007).

DOR function, expression and transport increase under chronic pain or stress circumstances (Bie and Pan 2007; Gendron et al. 2007b; Wang et al. 2008). In the C fibers, a direct interaction between the substance P precursor, protachykinin, and DOR has been reported to be responsible for the transport of DOR to the plasma membrane in DRG neurons (Guan et al. 2005). Substance P release is therefore associated to an incorporation of DOR to the presynaptic membrane, which would modulate pain transmission. This modulatory mechanism after substance P release during pain stimulation would be absent in conditional DOR, and conditional Nav1.8^{+} DOR KO mice exacerbating the development and manifestations of chronic pain, such as neuropathic pain. Accordingly, we have found that the expression of DOR mRNA is increased in DRGs of wild-type but not mutant mice after CFA or PSNL. Together, these findings strength the idea that functional significance of DOR in nociceptive neurons gain relevance during the development of chronic pain, and that increased DOR function in these particular neurons underlies the analgesic efficacy of SNC80 observed in our study. Overall, the role of DOR is complex (Basbaum et al. 2009; Woolf 2009), and likely to evolve in situations of chronic pain.

In conclusion, our data demonstrate a fundamental role for DOR expressed by primary afferent Nav1.8^{+} fibers in the development of chronic pain, and support the existence of an endogenous DOR tone that would counteract pain under these pathological conditions. Because these peripheral receptors are essential for opioid analgesia, these studies also provide strong basis for the design of peripherally acting drugs devoid of

psychotropic liability for the treatment of the clinical manifestations of neuropathic pain.

CB2 cannabinoid receptors and neuropathic pain

The present results have shown the crucial role of CB_2 cannabinoid receptors in the development of neuropathic pain through an immune mechanism linked to glial activation, modification of INF-γ activity, and subsequent inducible nitric-oxide sintase (iNOS) and chemokine receptor 2 (CCR2) stimulation. Hyperalgesia and allodynia induced by sciatic nerve injury were enhanced in $CB_2^{-/-}$ mice, as revealed by a mirror image of pain in the contralateral side. These behavioural manifestations of neuropathic pain matched the changes induced in microglial and astrocyte activation, astrocytic IFNγ expression and other biochemical parameters related to the immune response. The opposite behavioral and histological manifestations of neuropathic pain were revealed after nerve injury in transgenic mice overexpressing CB_2 receptors under the control of a PrP promoter in the brain and the spinal cord. The decreased hyperalgesia, allodynia and spinal glial activation in these transgenic mice could be related to the strong overexpression of CB_2 receptors at the level of the spinal cord. These CB_2 receptors were mainly expressed in microglia cells and neurons, but they were absent in astrocytes, in agreement with previous works (Van Sickle et al. 2005; Romero-Sandoval et al. 2008). However, these mice also showed an enhanced expression of CB_2 receptors in supraspinal structures involved in pain transmission, such as the thalamus and the PAG matter, which could also participate in the decreased manifestations of neuropathic pain. The opposite phenotype revealed in transgenic mice overexpressing CB_2 receptors and $CB_2^{-/-}$ mice underlines the relevance of these receptors in mediating the manifestations of neuropathic pain.

Microglial activation has pro-inflammatory actions (Watkins et al. 2003), and although it is not involved in acute pain (Raghavendra et al. 2003), this activation is required for induction and development of several chronic pain situations (Raghavendra et al. 2003; Watkins et al. 2003). Astrocyte-released peptides have also been involved in the nociceptive hypersensitivity and enhanced ciclooxigenasa 2 (COX-2) expression induced after peripheral nerve injury (Watkins et al. 2001). Glial activators include chemokines that enhance pain sensation and are under the control of immune mediators, as well as several neuromodulators released by the nearby neurons, such as prostaglandins (Tanga et al. 2006). Interferon proteins represent crucial modulators of the central and peripheral immune response (Bach et al. 1997), and the enhanced induction of INF-γ genes in $CB_2^{-/-}$ mice could participate in their nociceptive hypersensitivity. Indeed, a prolonged spinal increase in INF-γ levels in inflammatory responses in diseases such as viral infections and multiple sclerosis is thought to contribute to the associated persistent pain states. Indeed, INF-γ treatments in cancer therapy can result in spontaneous pain in humans (Quesada et al. 1986; Mahmoud et al. 1992), and intrathecal INF-γ administration in mice can cause pain-related behaviours in normal, but not in INF-γ receptor KO mice (Robertson et al. 1997). Moreover, it was demonstrated that intrathecal administration of INF-γ activates microglia through INF-γ receptors and it is sufficient to cause allodynia (Tsuda et al. 2009). Although the molecular and cellular mechanisms involved in interferon-induced pain remain largely unclear, it has been demonstrated that INF-γ can cause spontaneous firing of dorsal horn neurons in-vitro and in-vivo, as well as enhanced wind-up responses to electrical stimulation (Vikman et al. 2003; Vikman et al. 2005; Vikman et al. 2007). Interestingly, the pharmacological activation of CB_2 receptors suppresses wind-up responses of spinal nociceptive neurons and this

effect was more pronounced in the presence of pathological pain (Nackley et al. 2004).

On the other hand, we identified three α-interferon (INF-α) inducible genes (Ifit1, Ifit3 and IFi27), each of which was more strongly activated in $CB_2^{+/+}$ animals, when compared to $CB_2^{-/-}$ mice. INF-α responses caused by the sciatic nerve injury were reduced in the absence of CB_2 receptors. This blunted INF-α response may also contribute to the exacerbated neuropathic pain in $CB_2^{-/-}$ mice because centrally administered INF-α has antinociceptive effects (Hori et al. 1998). There is considerable evidence to suggest that some of the analgesic effects of INF-α are mediated by an interaction with the endogenous opioid system because the INF-α antinociception is naloxone-reversible, and INF-α is known to activate KOR and DOR (Jiang et al. 2000).

The enhanced IFN-γ response revealed by microarray data in $CB_2^{-/-}$ mice exposed to nerve injury has an important functional relevance *in vivo*. Thus, a direct relationship between the enhanced IFN-γ response and the neuropathic pain manifestations of $CB_2^{-/-}$ mice was demonstrated by using double KO mice deficient in CB_2 receptors and IFN-γ. The behavioural manifestations of neuropathic pain showed by the $CB_2^{-/-}$ mice were completely abolished in these double KO animals. IFN-γ is a crucial modulator of the central and peripheral immune responses suggesting that an alteration of the immune response seems to underline the neuropathic pain responses in $CB_2^{-/-}$ mice.

The manifestations of neuropathic pain observed in $CB_2^{-/-}$ mice and double KO mice deficient in CB_2 and IFN-γ suggest that endocannabinoids play an important role in the control of the immune

responses leading to the development of neuropathic pain. In support of this hypothesis, an enhancement in the levels of the two main endocannabinoids, anandamide and 2-AG, was revealed after sciatic nerve injury in the spinal cord and several brain areas involved in pain, such as the PAG matter and the RVM (Petrosino et al. 2007). The endocannabinoid levels were also enhanced after sciatic nerve injury in the DRG (Mitrirattanakul et al. 2006) and the section of the sciatic nerve proximal to the lesion (Agarwal et al. 2007). These increased endocannabinoid levels are likely related to enhanced biosynthesis or decreased catabolism and transport since endocannabinoids are produced on demand without any substantial storage (Di Marzo et al. 1994). Therefore, endocannabinoids could produce a tonic activation of CB_2 receptors after sciatic nerve injury that would limit the immune responses leading to the development of neuropathic pain. In agreement, both mechanical and thermal hyperalgesia produced after sciatic nerve injury were attenuated by the administration of N-arachidonoyl-serotonin, an inhibitor of FAAH, the enzyme responsible of the degradation of anandamide (Maione et al. 2007), which further support the role of endocannabinoids in the modulation of neuropathic pain.

Our hypothesis was demonstrated by replicating the enhanced behavioral and histological manifestations of neuropathic pain observed in $CB_2^{-/-}$ mice in irradiated $CB_2^{+/+}$ mice receiving bone marrow transplantation from $CB_2^{-/-}$. Thus, irradiated $CB_2^{+/+}$ mice receiving transplantation of bone marrow cells lacking CB_2 receptors showed enhanced manifestations of neuropathic pain as revealed by allodynia, hyperalgesia, and astrocytic and microglial cell activation in the contralateral side after sciatic nerve injury. By generating bone marrow-chimaeric mice, we have presented evidence that newly recruited monocytes from the bone marrow of $CB_2^{-/-}$ mice reconstitute the spinal cord efficiently by differentiating into

microglial cells. Most of the reconstituted cells after bone marrow transplantation are microglia (Toth et al. 2007). Therefore, our findings demonstrate a main role of the immune responses mediated by microglia in the development of increased neuropathic pain sensitivity in the $CB_2^{-/-}$ animals.

Subsequent to nerve injury, chemokine gradients formed by the chemokine ligands (CCL) 2 and 3 orchestrate the recruitment and activation of resident and monocyte-derived microglia via signaling through their respective receptors CCR2, CCR1 and CCR5 (Scholz and Woolf 2007). In particular, CCR2 expression in either resident microglia or bone marrow-derived macrophages may be sufficient for the development of mechanical allodynia in a murine neuropathic pain model (Zhang et al. 2007). CB_2 cannabinoid receptor activity may critically influence the induction of CCR2 expression by monocytes and thus inhibit their chemotaxis (Steffens et al. 2005). Moreover, endocannabinoids were found to abolish microglia activation by inhibiting NO release trough a mechanism linked to the MAPK pathway (Ullrich and Bachschmid 2000). Our data revealed that IFN-γ treatment of mouse BV-2 microglial cells evoked marked microglial activation as indicated by induction of iNOS and CCR2 gene expression. CB_2 activation however, significantly decreased the expression of these two IFN-γ inducible genes in BV-2 cells. These data suggest that CB_2 receptor signaling exerts anti-inflammatory effects in the neuropathic response by controlling IFN-γ mediated microglial activation and recruitment. Therefore, these data complement our *in vivo* observations in the neuropathic response in $CB_2^{-/-}$ mice.

Our double immunohystochemical analysis revealed that IFN-γ was mainly expressed in astrocytes after sciatic nerve injury and the astrocytic expression of IFN-γ matched the pattern of nociceptive hypersentivity in all the experiments. IFN-γ also seems to be present in neurons, but it is absent in the microglia cells. The presence of INF-γ receptor in microglia cells has been recently described (Tsuda et al. 2009). Previous studies have reported the presence of CB_2 receptors in microglia cells (Romero-Sandoval et al. 2008) and neurons (Van Sickle et al. 2005). In agreement, our studies have revealed that CB_2 receptors are expressed in microglial cells and neurons in the transgenic mice overexpressing CB_2 receptors. Taken together all these data, we can postulate a possible mechanism to explain the inhibitory modulation of neuropathic pain through the activity of CB_2 receptors (Figure 10). Thus, the neuroinflammatory process leading to the development of neuropathic pain seems to be initiated by the microglial activation produced after nerve injury (Scholz and Woolf 2007). However, this initial process required an activation of astrocytes in order to consolidate the progression and to develop a chronic pain state (Zhang et al.). The activated astrocytes would release IFN-γ which promote the consolidation of the neuroinflamatory process and spread this process to new microglial cells. IFN-γ seems to be also released by neurons under these conditions, which could additionally contribute to the initiation and consolidation of the neuropathic pain. IFN-γ promotes microglia activation by the induction of several inflammatory pathways, including an enhancement in iNOS and CCR2 activity. CB_2 receptors that are mainly located in the microglia cells would play a crucial role to control and limit the spreading of this neuroinflamatory process. Thus, the activity of CB_2 receptors in microglial cells would diminish the activation of these cells during neurophatic pain by inhibiting the expression of iNOS and CCR2. CB_2 receptors located in neurons could also participate

in the neuropathic pain response by decreasing the production of IFN-γ (Figure 10). These inhibitory effects would restrict the activation of microglia cells and would attenuate the development of neurophatic pain.

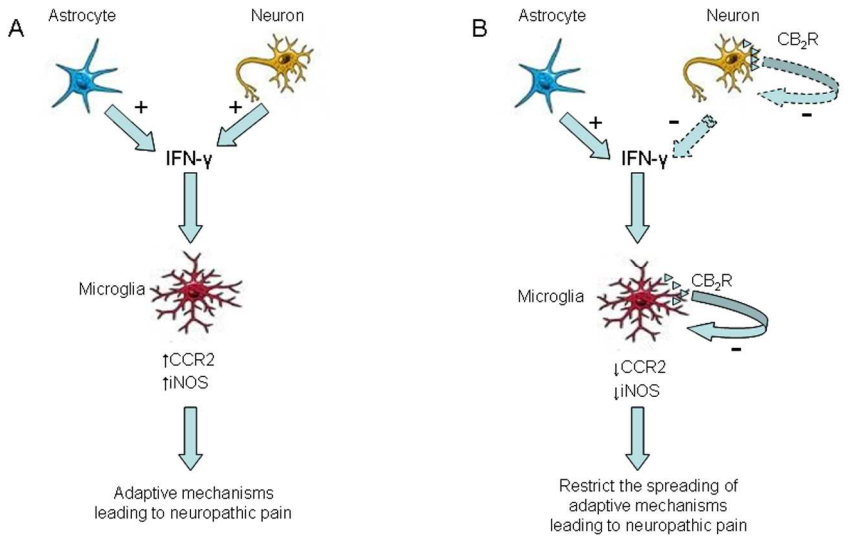

Figure 10: Hypothetical mechanism to explain the modulation of neuropathic pain through CB_2 receptor activation. The release of IFN-γ by activated astrocytes and neurons plays an important role in the neuroinflammatory process leading to the development of neuropathic pain. IFN-γ promotes microglia activation by the induction of several inflammatory pathways, including an enhancement in iNOS and CCR2 activity. The activated microglia promotes consolidation and progression of the neuropathic pain state. CB_2 receptors on microglial cells would control and limit the spreading of this neuroinflammatory process. Thus, the activity of CB_2 receptors in microglial cells would reduce the activation of these cells during neuropathic pain by regulating the expression of iNOS and CCR2. CB_2 receptors located in neurons could also participate in the neuropathic pain response by decreasing the production of IFN-γ. These inhibitory effects would restrict the activation of microglial cells and attenuate the development of neuropathic pain. In the absence of CB_2 receptors, IFN-γ would produce a more widespread activation of microglial cells which would enhance the manifestations of neuropathic pain and would be responsible for the presence of a mirror image of pain in the contralateral side

In the absence of CB_2 receptors, IFN-γ would produce a more widespread activation of microglia cells which would enhance the manifestations of neuropathic pain and would be responsible for the presence of a mirror

image of pain in the contra lateral side. Therefore, our findings demonstrate a crucial role of the immune response mediated by microglia in the development of increased neuropathic pain sensitivity in the $CB_2^{-/-}$ animals. A direct action on peripheral CB_2 receptors located in primary afferent neurons (Wotherspoon et al. 2005; Beltramo et al. 2006) and in keratinocytes involved in the release of β-endorphins (Ibrahim et al. 2005) has been reported to be involved in the analgesic responses of CB_2 agonist. However, these peripheral effects of CB_2 receptors do not seem to be involved in the enhanced manifestations of neuropathic pain observed in the present study in CB_2 receptors KO mice.

The interpretation of the results obtained in mice genetically modified either by increasing or eliminating specific genes may be limited by the fact that this genetic change could affect not only the target gene, but also other biological components, perhaps participating in the effects evaluated in these genetic models. Therefore, pharmacological studies using selective ligands of CB_2 receptors would be useful to confirm the relevance of the present results. Nevertheless, these genetic manipulations have been considered a key approach to the identification of alterations associated to different pathological conditions and to the discovering of new potential therapeutic targets in a variety of neuropsychiatric disorders. In this study, the use of different lines of genetically modified mice and the according results obtained in all these lines further support the relevance of the findings.

Our findings also reveal the crucial role played by the CB_2 receptor in the modulation of central immune responses during neuropathic pain. In contrast, the genetic disruption of the CB_1 receptor had no major consequences on the development of neuropathic pain (Castane et al. 2006) in spite of the high expression of these receptors in the CNS (Tsou

et al. 1998). Previous studies have used the Nav1.8-Cre line to target Nav1.8$^+$ neurons genetically, and analyze the role of CB1 receptors in these specific neurons in pain processing. The Nav1.8-targeted genetic ablation of CB_1 receptors (Agarwal et al. 2007) demonstrated the antinociceptive activity in both inflammatory and neuropathic pain produced by the activation of this receptor at the level of Nav 1.8$^+$ neurons.

The involvement of CB_2 receptors that have a low expression in neurons (Van Sickle et al. 2005) further emphasizes the relevance of these receptors in the central immune adaptive mechanisms leading to neuropathic pain. Therefore, CB_2 cannabinoid agonists could represent a new group of pharmacological agents for the treatment of neuropathic pain with decreased psychoactive side effects.

CONCLUSIONS

1. A significant enhancement of thermal hyperalgesia and mechanical and thermal allodynia was observed in DOR knockout mice exposed to a partial sciatic nerve ligation.

2. An enhancement of mechanical allodynia after partial sciatic nerve ligation or complete Freund adjuvant administration was observed in DOR conditional knockout mice that lack DOR in peripheral Nav1.8-positive primary nociceptive neurons.

3. Peripheral DOR tonically inhibit mechanical allodynia under conditions of both inflammatory and neuropathic pain, and are mandatory for delta agonist-induced analgesia in these models of chronic pain.

4. DOR could represent an interesting pharmacological target to develop new drugs with less psychotropic side-effects than classical opioids for the treatment of the clinical manifestations of neuropathic pain.

5. Hyperalgesia, allodynia and spinal microglial and astrocytic activation induced by sciatic nerve injury were enhanced in $CB_2^{-/-}$ mice, as revealed by a mirror image of pain in the contralateral side of the nerve injury.

6. Decreased hyperalgesia, allodynia and spinal glial activation were observed in the transgenic mice that overexpress CB_2 receptors.

7. The $CB_2^{-/-}$ bone marrow chimeric mice mimic the enhanced manifestations of neuropathic pain of the $CB_2^{-/-}$ mice, as revealed by allodynia, hyperalgesia, and astrocytic and microglial cell activation in the contralateral side of the spinal cord after sciatic nerve injury.

8. The enhanced induction of IFN-γ related genes revealed by microarray experiments in $CB_2^{-/-}$ mice could participate in their nociceptive hypersensitivity during neuropathic pain and could have an important functional relevance in vivo. In agreement, the enhanced manifestations of neuropathic pain revealed in $CB_2^{-/-}$ mice were completely abolished in the CB2/IFN-γ double KO animals.

10. The immunofluorescence analysis revealed that IFN-γ was mainly expressed in neurons and astrocytes, but was absent in microglia after sciatic nerve injury. This expression of IFN-γ matched the pattern of nociceptive hypersentivity in all experiments.

11. IFN-γ treatment of mouse BV-2 microglial cells evoked marked microglial activation as indicated by induction of iNOS and CCR2 gene expression. CB_2 activation decreases the expression of these two IFN-γ inducible genes in BV-2 cells.

12. IFN-γ would produce a more widespread activation of microglial cells, which enhance the manifestations of neuropathic pain, and would be responsible for the presence of a mirror image of pain in the contralateral side.

13. The present results reveal a crucial role of the CB_2 cannabinoid receptors in the control of neuropathic pain through an immune mechanism linked to glial activation and IFN-γ activity.

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APPENDIX

Appendix 1

Farmacología general del sistema endocannabinoide.

Xavier Nadal y Josep-Eladi Baños.

Dolor 2005;20(4): 199-212.

Appendix 2

A_{2A} adenosine receptor regulates glia proliferation and pain after peripheral nerve injury.

S. Andreea Bura, Xavier Nadal, Catherine Ledent, Rafael Maldonado and Olga Valverde.

Pain 2008 140(1); 95–103.

Appendix 3

Los Cannabinoides.

Xavier Nadal y Josep-Eladi Baños.

En Aliaga L, Baños JE, Barutell C, Molet J, Rodríguez de la Serna A (eds.). Tratamiento del Dolor Teoría y Práctica. 3ª ed. Publicaciones Permanyer. 2009;pag: 99-103.

Appendix 4.

Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury.

Beatriz de la Puente, Xavier Nadal, Enrique Portillo-Salido, Ricard Sánchez-Arroyos, Sergio Ovalle, Gabriel Palacios, Asunción Muro, Luz Romero, José Manuel Entrena, José Manuel Baeyens, José Antonio López-García, Rafael Maldonado, Daniel Zamanillo and José Miguel Vela.

Pain 2009, 145(3); 294–303.

Appendix 5

Pharmacological activation of 5-HT7 receptors reduces nerve injury-induced mechanical and thermal hypersensitivity.

Alex Brenchat, Xavier Nadal, Luz Romero, Sergio Ovalle, Asunción Muro, Ricard Sánchez-Arroyos, Enrique Portillo-Salido, Marta Pujol, Ana Montero, Xavier Codony, Javier Burgueño, Daniel Zamanillo, Michel Hamon, Rafael Maldonado and José Miguel Vela.

Pain 2010, 149(3);483–494.

Appendix 6

Sistemas opioides y control del dolor.

Josep-Eladi Baños y Xavier Nadal

En Zarranz JJ (ed.): Neurofarmacología contemporánea. Barcelona: Elsevier, 2011; 141-168.