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INVOLVEMENT OF THE OPIOID SYSTEM AND BDNF IN NEUROPLASTIC ALTERATIONS OCCURRING IN NEUROPATHIC PAIN CONDITIONS

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ABSTRACT

ABBREVATIONS

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Chronic pain affects approximately one in five adults, resulting in a greatly reduced quality of life and a higher risk of developing co-morbidities such as depression.

Among different forms of persistent pain, neuropathic pain represents a difficult task for therapy and basic research. Despite recent rapid development of neuroscience and modern techniques related to drug discovery, effective drugs based on clear basic mechanisms are still lacking. A deepened understanding of the molecular mechanisms underlying chronic pain states is needed for the discovery of new and improved therapies.

Neuropathic pain is triggered by lesions to the somatosensory nervous system that alter its structure and function so that pain occurs spontaneously and responses to noxious and innocuous stimuli are pathologically amplified. The pain is an expression of maladaptive plasticity within the nociceptive system, a series of changes that constitute a neural disease state. Multiple alterations distributed widely across the nervous system contribute to complex pain phenotypes. These alterations include ectopic generation of action potentials, facilitation and disinhibition of synaptic transmission, loss of synaptic connectivity and formation of new synaptic circuits, and neuroimmune interactions.

The endogenous Opioid System is probably the most important system involved in control of nociceptive transmission. Both the dynorphin and nociceptin systems have suggested as key mediators of some aspects of chronic/neuropathic pain. Beside these system, an important role for the brain derived neurotrophic factor (BDNF) has been recently suggested since its involvement in the peripheral and central sensitization phenomena is known.

Based on these evidences, here we studied neuroplastic alterations occurring in chronic pain conditions, in mice subjected to the right chronic constriction injury (CCI). First we investigated the gene expression alterations of the both BDNF and Opioid System in spinal cord (SC) and dorsal root ganglia (DRGs) of injured mice at different intervals of time. A transient up-regulation of pBDNF and pDYN was observed in SC, while increasing up-regulation of ppN/OFQ was found in the DRGs of injured mice compred to controle ones.

Based on the growing importance attributed to the cerebral neuroplastic alterations we also investigated in the same experimental conditions gene expression changes of these systems in different brain areas fourteen days after surgery. We found gene expression alterations in several areas of CCI mice. We observed up-regulation of pBDNF mRNA levels in the amygdala and in the prefrontal and anterior cingulate cortices, while a down-regulation of its gene expression in the thalamus. Moreover, an up-regulation of pDYN gene expression in the prefrontal and anterior cingulate cortices, and a dow-regulation of its mRNA in the brainstem was determined. Concerning nociceptin system, we found ppN/OFQ down-regulation in the brainstem, hippocampus, amygdala, nucleus accumbens and caudate putamen, and a down-regulation of NOP receptor in the brainstem and caudate putamen. In the same brain regions we also determined bioactive nociceptin peptide levels, and elevated N/OFQ levels were observed in the amygdale of the lesioned mice.

Recently, an increasing interest has been given to the elucidation of epigenetic mechanisms regulating gene expression changes in different pathological conditions, including pain states. Hence, we studied alterations of histone modification in the BDNF and DYN gene promoters, in the SC of CCI animals seven days after surgery. In particolar, our study revealed a significant decrease of H3K27me3 and H3K9me2, repressive marks, and of H3K4me3, activating marks, in the pDYN gene promoter region. No changes of histone modifications were observed in the pBDNF gene promoter region. In parallel, we also investigated BDNF involvement in neuropathic pain through the analysis of the behavioral signs of hyperalgesia and allodynia in a strain of mice partially lacking for BDNF, subjected to CCI. A similar development of hyperalgesia was observed in ICR/CD-1 and BDNF +/+ (wild type) mice. In contrast, a later onset of hyperalgesia was observed in BDNF +/- mice (BDNF lacking mice). In addition, BDNF +/- animals exhibited significantly lower signs of allodynia than BDNF +/+, in both the cold plate and the acetone tests.

In the last part of the present study, in order to provide a unique approach to explore receptor localization and function in vivo of the N/OFQ-NOPr system, I performed a preliminary characterization and validation of an innovative knock-in mouse strain NOP-EGFP mice where enhanced Green Fluorescent Protein is attached on the C-terminal of the NOP receptor. We observed that N/OFQ somministration was able to

induce NOP receptor internalization in primary neurons. Analysis on brain slices showed that the receptors signalappear to be located in brain regions that have been previously demonstrated to contain NOP receptor mRNA and NOP receptor binding sites. This model can be useful for a better characterization of pathophysiological role of this system in neuropathic pain. The characterization of the NOP-EGFP mice could provide a unique technology useful to answer different questions concerning the involvement of N/OFQ-NOPr system in chronic pain condition.

Overall, our results could be important to partially fill the lack of knowledge of which and how neuropeptidergic systems are involved in neuroplastic mechanism occurring in neuropathic pain that lead to establish the chronic condition and suggest the possibility of using drugs acting on these systems for the treatment of this invalidating disease.

ABBREVATIONS

ACCx	anterior cingulate cortex
Amy	amygdala
BS	brainstem
CCI	chronic constriction injury
CNS	Central nervous system
CP	caudate putamen
DDCt	Delta-Delta Ct
DRG	dorsal root ganglia
DYN	dynorphin
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hippo	hippocampus
Hyp	hypothalamus
N/OFQ	nociceptin
NAc	nucleus accumbens
NMDA	N-methyl-D-aspartate
NOP	NOP receptor
ORL1	orphanin receptor like 1
pBDNF	proBDNF
pDYN	prodynorphin
pEKN	proenkephalin
PFCx	prefrontal cortex
pKOP	pro kappa opioid receptor
PMOC	proopiomelanocortin
pNOP	pro NOP receptor
ppN/OFQ	prepronociceptin
SC	Spinal cord
SSCx	somatosensory cortex
Th	thalamus

1. INTRODUCTION

1.1 Neurobiological bases and types of pain

According to the definition of IASP (International Association for the Study of Pain - 1986) and according to the association of O.M.S, "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."

The pain consists of several components: the physiological-discriminative, the emotional-affective and the cognitive-evaluative. Although the emotional and cognitive mechanisms influence the pain perception, the physiological-discriminative component is the most important especially for the pharmacological therapy.
The nociceptive stimuli are collected in the periphery from specialized free synaptic terminals, the nociceptors. The nociceptors represent the distal part of the nerve fibers that are classified according to their velocity of conduction and the type of stimulus that activates them. There are two types of fiber: Aδ fibers, fast-conducting (6-30 m/s), myelinated, activated by mechanical and thermal stimuli and C fibers, unmyelinated, slow-conducting (<2m / s), whose free terminals are represented by polymodal nociceptors. These fibers have the neuronal soma in the dorsal root ganglia (DRG) and represent the first-order neurons; their anatomical structure (T-cells) allows the impulse transmission to a second-order neuron located in the dorsal horn of the spinal cord. The afferent fibers of these neurons intersect and run along the entire spinal cord ascending through the contralateral portion of the medulla; these fibers convey information to specific thalamus nuclei where they synapse with the third order neurons which project to cortical areas such as somatosensory, anterior cingulate and insular cortices. This spino-thalamic tract is defined as the ascending pathway of pain control (Fig. 1).

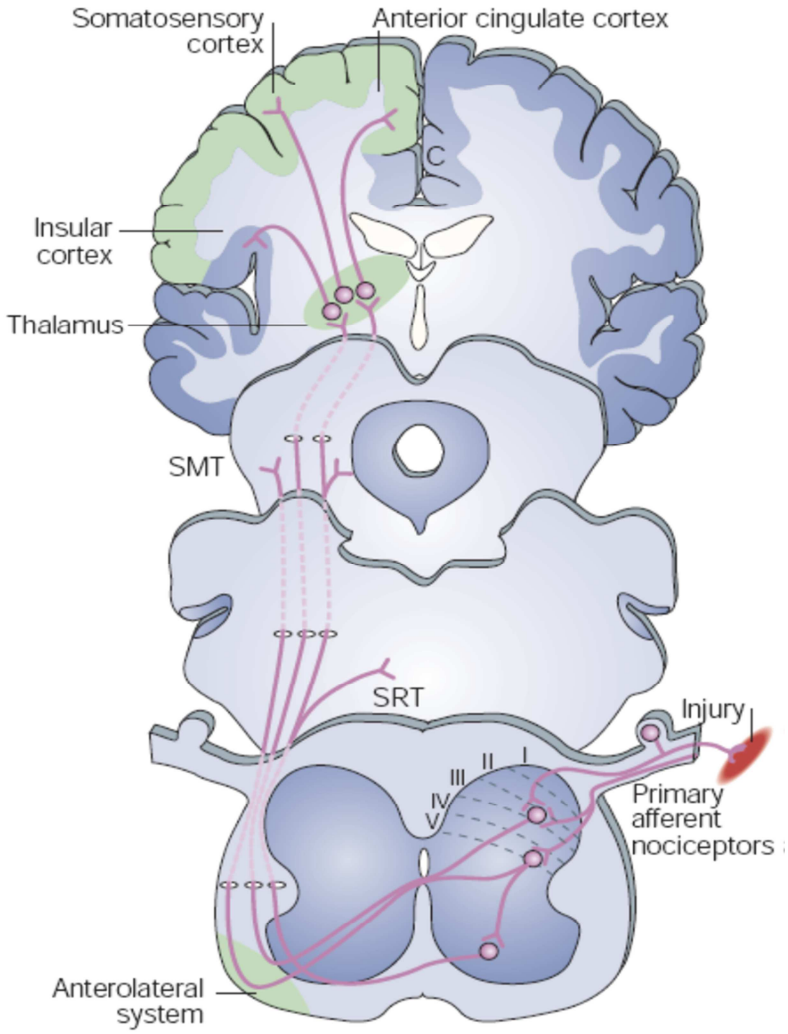

Figure 1. Schematic representation of the ascending path of pain impulses transmission.

The functional activity of the ascending pathways above described is modulated by the descending control system (Fig. 2). This system is influenced by higher brain structures (such as thalamus, amygdala and anterior cingulate cortex) and originates from the periaqueductal gray (PAG), at the midbrain level, and the raphe medullary and pontine nuclei. Monoaminergic fibers from these nuclei (5-HT, NA) run along the dorsal-lateral tracts of the spinal cord and terminate in the laminae of the spinal cord. These fibers control the first-order neuron activity, either directly or through the activation of an inhibitory interneuron, and are also able to modulate the second order neurons activity through postsynaptic receptors activation by reducing the excitability. Overall, the pain control descending patways is able to produce a reduction of the peripheral nociceptive input *(Carbone E. et al. 2009).*

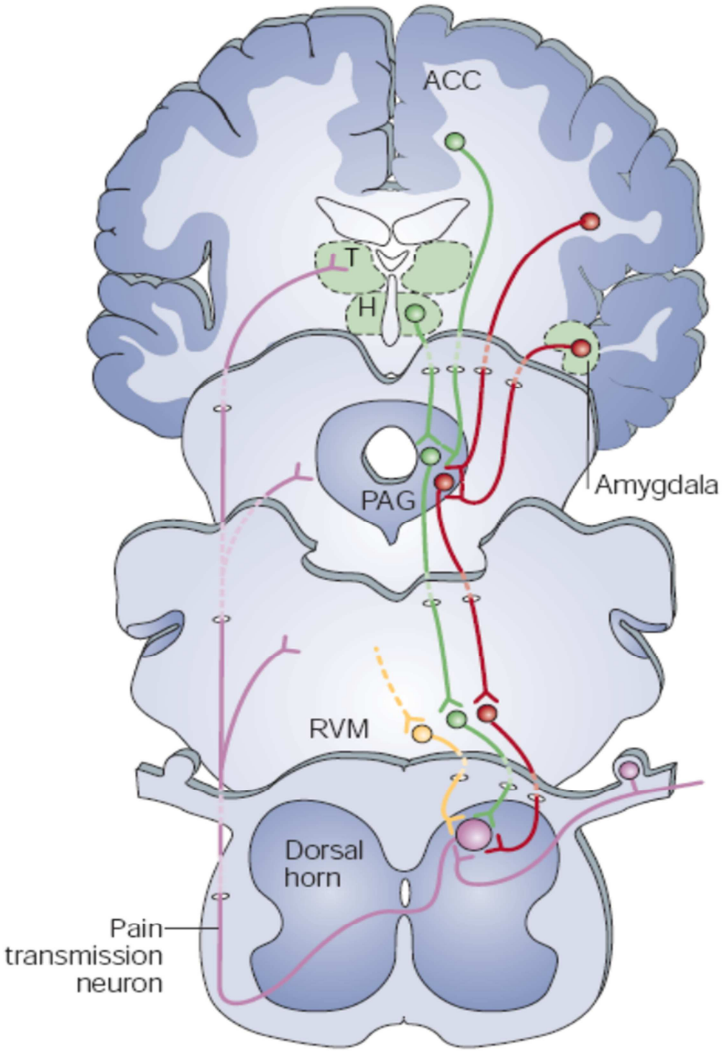

Figure 2. Schematic representation of the descending path of pain impulses transmission.

Concerning type of pain, there are four main categories of pain more or less similar to each other.

Nociceptive pain is a type of pain which all people have experienced. It is an alarm mediated by high-threshold unmyelinated C or thinly myelinated Aδ primary sensory neurons that feed into nociceptive pathways of the central nervous system (CNS). These nociceptor neurons express specialized transducer ion channel receptors, mainly transient recep- tor potential (TRP) channels, tuned to respond to intense thermal or mechanical stimuli as well as exogenous and endogenous chemical mediators. For nociceptive pain to subserve its protective function, the sensation must be so unpleasant that it cannot be ignored. Nociceptive pain occurs in response to noxious stimuli and continues only in the maintained presence of noxious stimuli. It alerts us to external stimuli, such as pinprick or excessive heat, and internal stimuli, such as myocardial ischemia in patients with coronary artery disease. Certain diseases may generate recurrent or ongoing noxious stimuli to produce chronic nociceptive pain as osteoarthritis. *(Costigan M. et al. 2009).*

Inflammatory pain occurs in response to tissue injury and the subsequent inflammatory response. Here the imperative shifts from protecting the body against a potentially damaging noxious stimulus to addressing the consequences of damage. To aid healing and repair of the injured body part, the sensory nervous system undergoes a profound change in its responsiveness; normally innocuous stimuli now produce pain and responses to noxious stimuli are both exaggerated and prolonged. Heightened sensitivity occurs within the inflamed area and in contiguous noninflamed areas as a result of plasticity in nociceptors and central nociceptive pathways *(Costigan M. et al. 2009).*

Dysfunctional pain is caused by a malfunction of the somatosensory apparatus and this malfunction can be considered a disease in its own right. Dysfunctional pain occurs in situations in which there is no identifiable noxious stimulus nor any detectable inflammation or damage to the nervous system. It is unclear in most cases what causes the manifestation or persistence of dysfunc- tional pain. This syndrome share some features of neuropathic pain *(Costigan M. et al. 2009).*

Neuropathic pain is a chronic pain state caused by a lesion or disease affecting the somatosensory system alters nociceptive signal processing so that pain is felt in the

absence of a stimulus, and responses to innocuous and noxious stimuli are enhanced *(Costigan M. et al. 2009).* It can be divided into central or peripheral neuropathic pain based on the anatomical location of the injury or disease: peripheral nervous system (PNS, eg. Peripheral nerves, dorsal root ganglia (DRG) and dorsal root) and central nervous system (CNS, eg . spinal cord and thalamus).

1.2 Neuropathic Pain

Neuropathic pain is caused by pathology involving directly or indirectly the nervous system. The pathology can be due to diseases that involve the nervous system (e.g., AIDS and diabetes), to dysfunction of the nervous system (e.g., following stroke), or to a traumatic injury of the nervous system. The pathology can involve the peripheral nervous system, the central nervous system, or both.

Nerve trauma can occur in the peripheral or the central nervous system. Peripheral lesions can be caused by nerve compression, limb amputation, or traumatic injuries and commonly result in dysesthesias, paresthesias, or chronic neuropathic pain. After limb amputation, painful sensations may be perceived as originating in the missing part of the body *(Casale R. et al. 2009)*. Pain of central origin may occur following spinal cord or brain trauma due to injury, compression (by tumors or cysts), or hemorrhagic or ischemic stroke. Not all central nervous system traumas result in central pain, but a necessary condition is damage to the spinothalamic tract. Moreover, it appears that spinal cord injury induces pain only if the gray matter is damaged.

Various disease states can lead to the development of neuropathic pain. Diabetes can often lead to a painful peripheral neuropathy. Diabetic neuropathy is a length-dependent neuropathy because it most commonly affects the extremities and progresses from more distal to proximal levels. High intracellular glucose levels can lead to pathological changes, such as vasoconstriction endothelial hyperplasia, and increased oxidative stress. These changes in turn promote neuronal dysfunction through decreased nerve blood flow and consequent neural hypoxia. Chronic alcohol abuse may also result in a peripheral neuropathy in the lower extremities due to

direct degenerative effects on both myelinated and unmyelinated nerve fibers, disruption of axonal transport, and enhancement of glutamate neurotoxicity in neurons.

Painful neuropathies can also develop as a result of viral infections. For example, after an acute infection of chicken pox, the neurotropic human herpes virus, varicella-zoster, remains dormant in the dorsal sensory or cranial nerve ganglia. A localized reoccurrence of the varicella-zoster virus (herpes zoster or shingles) can occur particularly when the immunesystem is suppressed, as is common in older adults or individuals with other immunosuppressive conditions such as Hodgkin's disease or HIV. This is most likely to occur in the dermatomes that had been most severely affected by the primary disease. The acute rash and pain are restricted to the area of one or several dermatomes and mos commonly occur in the chest and trunk. After the acute viral infection subsides, approximately one-third of patients develop a chronic pain condition known as postherpetic neuralgia (PHN). The pain is described as burning, tingling, deep aching, itching, or stabbing. The pain in a subset of PHN patients is thought to be due to 'irritable nociceptors.' These patients report severe mechanical hyperalgesia but have minimal sensory loss in the painful area and their responses to thermal stimulation remain normal.

HIV infection commonly leads to the development of neuropathy either by immune system and macrophage-mediated axonal degeneration or by toxic effects of antiviral treatment medications on mitochondria. Similar to diabetic neuropathy and amyloidosis, HIV-induced neuropathy characteristically progresses by first affecting the most distal portions of axons. Activation of proinflammatory mediators such as tumor necrosis factor-a (TNF-α), interleukin (IL)-6, and nitric oxide in the dorsal root ganglia (DRG) is thought to contribute to the axonal damage and to the development of a painful neuropathy frequently characterized by bilateral painful paresthesias, dysesthesias, or numbness. Autoimmune diseases such as Guillaine–Barre´ syndrome and multiple sclerosis (MS) cause demyelination and sensory deficits including pain. Approximately 80% of Guillaine–Barre´ and 50% of MS patients report pain. The etiology of MS is not well-known, although it is believed that the onset of the disease involves pathology of the immune system, particularly disruption of myelin proteins. Guillaine–Barre´ syndrome has several subtypes based

on the suggested etiology of the disease, namely acute inflammatory demyelinating polyradiculoneuropathy, acute motor axonal neuropathy, and acute motor and sensory axonal neuropathy. T cell disruption of myelin proteins is indicative of the first subtype, whereas antibodies to gangliosides are thought to promote demyelination and injury in the other two subtypes.

At the end chemotherapeutic drugs such as vincristine or paclitaxel promote microtubule stabilization, thus inhibiting tumor cell proliferation. However, the effectiveness of chemotherapy agents is often limited by severe neurotoxic side effects. Many patients undergoing paclitaxel chemotherapy develop peripheral neuropathies *(Borzan J., Meyer R.A. 2009).*

Once neuropathic pain is generated, the sensory hypersensitivity typically persists for prolonged periods, even though the original cause may have long since disappeared, as after nerve trauma. The syndrome can nevertheless progress if the primary disease, such as diabetes mellitus or nerve compression, continues to damage the nervous system. Neuropathic pain is not an inevitable consequence of neural lesions, though. On the contrary, the pain associated with acute neural damage usually transitions to chronic neuropathic pain in a minority of patients *(Costigan M. et al. 2009).*

For damage of a relatively small nerve, such as the ilioinguinal nerve during hernia repair, the risk of persistent (more than two years) pain is on the order of ~5% *(Kalliomaki M.L. et al. 2008),* whereas sectioning a large nerve, such as the sciatic nerve or multiple intercostal nerves during thoracotomy, produces sustained neuropathic pain in 30%–60% of patients *(Ketz A.K. 2008, Maguire M.F. et al. 2006).* Understanding why one individual develops chronic pain and another with an effectively identical lesion is spared is obviously crucial to developing strategies to abort such transitions *(Costigan M. et al. 2009).*

Epidemiological studies on the prevalence of neuropathic pain indicate a high incidence (~5%) *(Bouhassira D. et al. 2008).* Associated risk factors include gender, age, and anatomical site of the injury. Emotional and cognitive factors can also influence how patients react to chronic pain, but it is much less certain if these factors contribute to the risk of developing pain *(Costigan M. et al. 2009).*

1.2.1 Neuropathic Pain symptoms

Patients typically have paradoxical sensory perceptions with pain as a dominating positive symptom combined with lesion-induced reduced sensations. These perceptions are usually unique and have not been experienced before by patients. This coexistence of signs of hypersensitivity and hyposensitivity is quite common in neurological disorders; for example, when parkinsonian tremor develops after degeneration of the substantia nigra or when spasticity develops after spinal cord injury. However, by contrast with these motor disturbances, pain as a subjective sensory symptom is not visible, is diffi cult to measure, and involves not only physical aspects, but also psychological and emotional components. The characteristic sensory abnormalities are crucial findings to correctly diagnose neuropathic pain and to distinguish this from other pain types.

A lesion to a sensory or mixed peripheral nerve with a cutaneous branch, or damage to a central somatosensory pathway, characteristically leads to an area of sensory defi cit in the related innervation territory. These negative sensory signs can include a defi cit in the perception of mechanical or vibratory stimuli, which indicates damage to large diameter afferent fibres or to the dorsal column tract, and a loss of noxious and thermal percerption, which indicates damage to small diameter afferent fibres or to central pain processing pathways such as the spinothalamic tract. Electrophysiological techniques and nerve biopsy samples can be useful to help assess the attenuation of neuronal function and to document the extent of neuropathy. The important question in the management of patients with chronic pain is, however, whether their pain is caused by the neuronal lesion or whether other pain disorders dominate the clinical picture and coexist with a neuropathy. To diagnose neuropathic pain and distinguish it from nociceptive pain it is helpful to analyse the exact quality of somatosensory abnormalities. Patients with neuropathic pain almost always have areas of abnormal sensation or hypersensitivity in the affected area, which can be adjacent to or combined with skin areas of sensory deficit (tab. 1). These positive symptoms are paraesthesias (ie, skin crawling sensation or tingling), spontaneous (not stimulus-induced) ongoing pain, and shooting, electric shock-like sensations. Many patients with neuropathic pain also have evoked pain (ie, stimulus-induced

pain and hypersensitivity). Patients usually report mechanical and thermal hypersensitivity. Two types of hypersensitivity can be distinguished. First, allodynia is defi ned as pain in response to a nonnociceptive stimulus. In cases of mechanical allodynia, even gentle mechanical stimuli such as a slight bending of hairs can evoke severe pain. Second, hyperalgesia is defined as an increased pain sensitivity to a nociceptive stimulus. Another evoked feature is summation, which is the progressive worsening of pain evoked by slow repetitive stimulation with mildly noxious stimuli, for example, pin pricks. In terms of clinical practice and research, the term allodynia is mainly reserved for pain induced by light moving stimuli (mechanical dynamic allodynia), whereas the term hyperalgesia is used for other forms of mechanically induced pain (Tab. 1) *(Baron R. et al. 2010).*

	Definition	Bedside assessment	Expected pathological response
Negative symptoms and signs			
Hypoaesthesia	Reduced sensation to non-painful stimuli	Touch skin with painter's brush, cotton swab, or gauze	Reduced perception, numbness
Pall-hypoaesthesia	Reduced sensation to vibration	Apply tuning fork on bone or joint	Reduced perception threshold
Hypoalgesia	Reduced sensation to painful stimuli	Prick skin with single pin stimulus	Reduced perception, numbness
Thermal hypoaesthesia	Reduced sensation to cold or warm stimuli	Contact skin with objects of 10°C (metal roller, glass with water, coolants such as acetone); contact skin with objects of 45°C (metal roller, glass with water)	Reduced perception
Spontaneous sensations or pain			
Paraesthesia	Non-painful ongoing sensation (skin crawling sensation)	Grade intensity (0–10); area in cm^2	..
Paroxysmal pain	Shooting electrical attacks for seconds	Number per time; grade intensity (0–10); threshold for evocation	..
Superficial pain	Painful ongoing sensation, often a burning sensation	Grade intensity (0–10); area in cm^2	..
Evoked pain			
Mechanical dynamic allodynia	Pain from normally non-painful light moving stimuli on skin	Stroke skin with painter's brush, cotton swab, or gauze	Sharp burning superficial pain; present in the primary affected zone but spreads beyond into unaffected skin areas (secondary zone)
Mechanical static hyperalgesia	Pain from normally non-painful gentle static pressure stimuli on skin	Apply manual gentle mechanical pressure to skin	Dull pain; present in the area of affected (damaged or sensitised) primary afferent nerve endings (primary zone)
Mechanical punctate, pin-prick hyperalgesia	Pain from normally stinging but non-painful stimuli	Prick skin with a safety pin, sharp stick, or stiff von Frey hair	Sharp superficial pain; present in the primary affected zone but spreads beyond into unaffected skin areas (secondary zone)
Temporal summation	Increasing pain sensation (wind-up-like pain) from repetitive application of identical single noxious stimuli	Prick skin with safety pin at intervals of <3 s for 30 s	Sharp superficial pain of increasing intensity
Cold hyperalgesia	Pain from normally non-painful cold stimuli	Contact skin with objects of 20°C (metal roller, glass with water, coolants such as acetone); control: contact skin with objects of skin temperature	Painful, often burning, temperature sensation; present in the area of affected (damaged or sensitised) primary afferent nerve endings (primary zone)
Heat hyperalgesia	Pain from normally non-painful heat stimuli	Contact skin with objects of 40°C (metal roller, glass with water); control: contact skin with objects of skin temperature	Painful burning temperature sensation; present in the area of affected (damaged or sensitised) primary afferent nerve endings (primary zone)
Mechanical deep somatic hyperalgesia	Pain from normally non-painful pressure on deep somatic tissues	Apply manual light pressure at joints or muscles	Deep pain at joints or muscles

..=not applicable. Reproduced from Baron,[1] with permission from Nature Publishing Group.

Table 1. Definition and assessment of negative and positive sensory symptoms and signs in patients with neuropathic pain *(Baron R. et al. 2010).*

1.2.2 Neuropathic Pain models

Animal models provide pivotal systems for preclinical studies of neuropathic pain and serve as an experimental basis for mechanistic investigations and testing new therapeutic interventions *(Colleoni M., Sacerdote P. 2010).*

The ideal models should result in reproducible sensorydeficits such as allodynia, hyperalgesia and spontaneous pain over a sustained period. Different types of animal models have been established to meet the diverse etiology and consequently the diverse manifestations of the neuropathy (*JaggiA.S. et al. 2008).*

Most of animal models of neuropathic pain were generated starting from the late 1980, using rat as preferred species. More recently pain models originally developed in rats have been transposed for use in mice; a strong motive for the use of mice is the availability of genetically characterized or manipulated inbred strains, particularly transgenic mouse lines in which specific proteins or signal transduction component have been altered throughout genetic knockout technology. In pain studies more than in other animal models of disease, particular care has to be given to the strains used, since a strong influence of genetic background on pain sensitivity exists *(Colleoni M., Sacerdote P. 2010).*

A common pitfall of all rodent models of neuropathic pain is the inappropriateness of the outcome measures utilized. In fact they focus on stimulus-evoked pain and hyperreflexia at a particular moment in time, whereas a high proportion of patients with neuropathic pain have ongoing, spontaneous pain and sensory loss. The recognition of spontaneous pain in experimental animals is particularly difficult. Weight loss, sleep disturbances, reduced movement, spontaneous paw lifting, scratching or shaking have all been accepted to reflect spontaneous pain *(Zimmermann M. 2001).*

The numerous models of neuropathic pain in mice (Tab. 2) can be classified five gross categories *(Colleoni M., Sacerdote P. 2010)*:

- central pain models
- peripheral nerve injury models
- models of disease-induced neuropathic pain
- iatrogenic (drug-induced) neuropathic pain, and inherited neuropathies

S. no.	Name of model	Principle of injury	Species
1	Axotomy (complete sciatic nerve transection)	Complete transection of sciatic nerve	Rats
2	Chronic constriction injury	Four loose ligatures around sciatic nerve	Rats, mice
3	Partial sciatic nerve ligation (Seltzer Model)	Tight ligation of one-third to half of sciatic nerve	Rats, mice
4	Spinal nerve ligation	(i) Tight ligation of L5, L6 spinal nerves (ii) tight ligation of L7 spinal nerve	Rats, *Macaca fascicularis*
5	Spared nerve injury	Axotomy of tibial and common peroneal nerves	Rats, mice
6	Tibial and sural nerve transection	Axotomy of tibial and sural nerves	Rats
7	Ligation of common peroneal nerve	Ligation of common peroneal nerve	Mice
8	Sciatic cryoneurolysis	Freezing of the sciatic nerve	Rats
9	Caudal trunk resection	Resection of caudal trunk	Rats, mice
10	Sciatic inflammatory neuritis	Injection of zymosan, HMG, TNF-alpha around sciatic nerve	Rats, mice
11	Cuffing-induced sciatic nerve injury	Implantation of polyethylene cuff around sciatic nerve	Rats, mice
12	Photochemical-induced sciatic nerve injury	Thrombosis in small vessels supplying sciatic nerve by photosensitizing dye and laser	Rats, mice
13	Laser-induced sciatic nerve injury	Radiation mediated reduction in blood supply to sciatic nerve	Rats
14	Weight drop or contusive spinal cord injury	Dropping a weight over the exposed spinal cord	Rats, mice
15	Excitotoxic spinal cord injury	Intraspinal injections of excitatory amino acids	Rats, mice
16	Photochemical spinal cord injury	Thrombosis in blood vessels supplying the spinal cord by photosensitizing dye and laser	Rats
17	Spinal hemisection	Laminectomy of T11–T12 segments.	Rats
18	Drugs-induced		
(a)	Anti-cancer agents (vincristine, cisplatin, oxaliplatin, paclitaxel)	Direct injury of drugs to the nerves of peripheral nervous system	Rats, mice, guinea pigs
(b)	Anti-HIV agents (2,3-dideoxycytidine, didanosine)		Rabbits, rats
19	Diabetes-induced neuropathy	Persistent hyperglycemia-induced changes in the nerves	Rats, mice
(a)	Streptozotocin-induced		
(b)	Genetic models		
20	Bone cancer pain models		
(a)	Femur, calcaneus,tibial, humerus bone cancer pain	Inoculation of cancerous cells into respective bones	Rats, mice
(b)	Neuropathic cancer pain	Growing a tumor in vicinity of sciatic nerve	Mice
(c)	Skin cancer pain	Injection of melanoma cells in plantar region of hind paw	Mice
21	HIV-induced neuropathy	Delivery of HIV-1 protein gp120 to sciatic nerve	Rats
22	Post-herpetic neuralgia		
(a)	Varicella Zoster virus	Injection of viral infected cells in the footpad	Rats, mice
(b)	Herpes simplex virus	Depletion of capsaicin-sensitive	
(c)	Non-viral model	Afferents with resiniferatoxin	Rats
23	Chronic ethanol consumption/withdrawal	Administration of ethanol over extended period (around 70 days)	Rats
24	Pyridoxine-induced	Administration of high dose pyridoxine for long period	Dogs, rats
25	Trigeminal Neuralgia	Compression of trigeminal ganglion chronic constriction injury to infra-orbital nerve	Rats Rats
26	Orofacial pain	Injection of formalin, carrageenan into temporomandibular joints and maxilla	Rats, mice
27	Acrylamide-induced	Administration of acrylamide for prolonged period	Rats

Table 2 List of different animal models of neuropathic pain *(Jaggi A.S. et al. 2008)*.

Central pain models mimic neuropathic pain resulting from CNS pathologies and represent a form of neuropathic pain that is associated with lesions of the brain or the spinal cord after a stroke or other traumatic injury *(Colleoni M., Sacerdote P. 2010).* Different models have been developed such as: *thalamic syndrome* resulting from stroke, based on a small hemorrhagic stroke lesion induced by collagenase injection in the ventral posterolateral nucleus of the rat thalamus *(Wasserman J. et al. 2009)*; spinal cord injury and contusive model caused by contusion or weight dropping, spinal cord compression, excitatory neurotoxins, photochemically induced ischemia, spinal cord hemisection, crushing of spinal cord *(Starkey M.S. et al. 2009)*; excitotoxic model based on intraspinal or intrathecally injection of some excitoxins, such as quisqualic acid or other excitatory aminoacids *(Fairbanks C.A. et al. 200)*; photochemical model based on intravenous injection of the photosensitising dye *(Gaviria M. et al 2002).*

Regarding peripheral nerve injury model nerve injury to induce hyperalgesia and allodynia in rodents, which are similar to the symptoms of neuropathic pain in humans. numerous models have been developed in rodents that generate a mixture of intact and injured fibres within a peripheral nerve, usually the sciatic, for ease of access and its relatively large size. Neuropathic pain behaviour is thought to be attributable to altered properties in the intact rather than the damaged fibres. Numerous types of nerve lesions have been employed including transection, resection, crush, complete or partial tight ligation, cryoneurolysis, and loose ligation with inflammatory materials (Fig. 3) *(Colleoni M., Sacerdote P. 2010).*

The type of nerve lesion can clearly influence the character of the ensuing neuropathic behaviours *(Colburn R.W. et al. 1999).*

Various partial nerve lesions have been developed experimentally to study neuropathic pain. Such models include chronic constriction injury of the sciatic nerve (CCI) *(Bennet G.J, Xie Y.K. 1988)*; partial sciatic nerve ligation (PSL) (Seltzer Z. et al. 1990) and spinal nerve ligation (SNL L5/L6) *(Kim S.H., Chung J.M. 1992)*, which represent three of the best-characterized rodent models of peripheral neuropathy (Fig. 3). These classic models produce similar pain behaviours with some variation in the magnitude *(Bennet G.J. 1993, Kim S.H. et al. 1997).* The model of CCI is one of the most commonly used models because it is reliable and easily reproducible, and due

to the presence of the constrictive ligatures, it combines nerve compression with Wallerian degeneration and an epineurial inflammatory lesion. This model in mice is produced by three loose ligatures that are tied around the common sciatic nerve exposed at the level of the mid-thigh, proximal to the trifurcation of the nerve. This procedure results in intraneural oedema, which strangulates the nerve, effectively axotomizing many but not all of the nerve axons. It results in the development of hyperalgesia to thermal stimulus and allodynia to mechanical stimuli. In contrast with CCI model that consists in the ligation of the total sciatic nerve, partial sciatic nerve injury that consists in the tight ligation of only 1/3 to 1/2 of the common sciatic diameter. Spinal nerve ligation consists of ligating unilaterally and tightly spinal nerves L5 and/or L6 of rodents at a location distal to the dorsal root ganglia and results in allodynia and hyperalgesia that develop quickly after ligation and last for at least 4 months *(Colleoni M., Sacerdote P. 2010).*

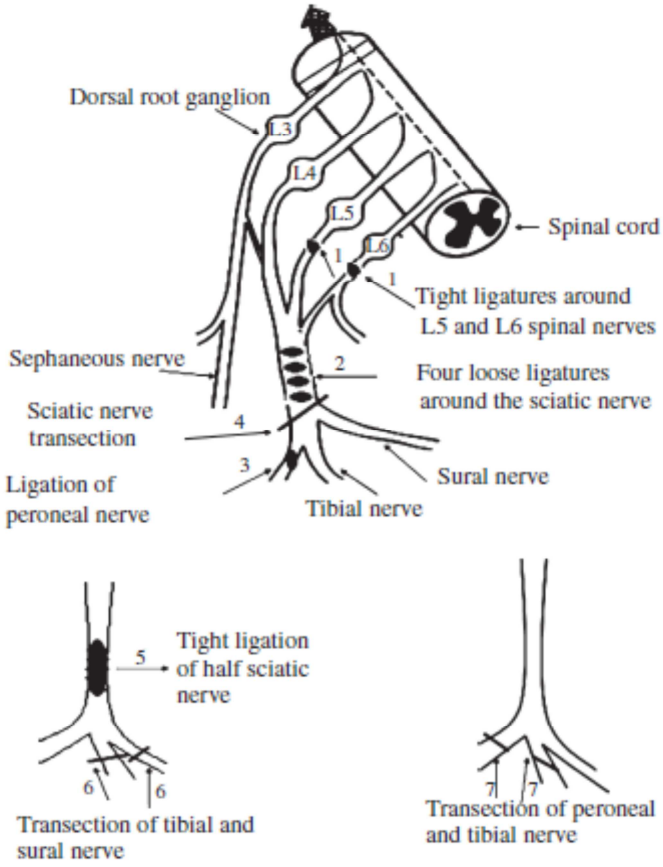

Figure 3 Representation of different peripheral nerve injury models (1) spinal nerve ligation by tightly ligating L5 and L6 spinal nerves; (2) chronic constriction injury by placing four loose ligatures around the sciatic nerve; (3) ligation of common peroneal nerve; (4) axotomy model by sciatic nerve transection; (5) partial sciatic nerve ligation by tight ligation of one-third to half of sciatic nerve; (6) tibial and sural nerve transection model by transaction of tibial and sural nerve; (7) spared nerve injury model by transaction of peroneal and tibial nerve *(Jaggi A.S. et al. 2008)*.

Regarding disease-induced neuropathic pain models trying to induce the disease that will thereafter cause the neuropathic damage, such as painful peripheral diabetic neuropathy, post-herpetic neuralgia, cancer-associated neuropathic pain, multiple sclerosis neuropathic pain, complex regional pain syndrome type I, HIV (and antiretroviral)-induced painful neuropathy. Iatrogenic (drug-induced) neuropathic pain models are obtained through animals Chemotherapy treatment. Finally, in recent years have been created inherited-induced neuropathies models due to the use of molecular clones of many membrane proteins localized in the axons myelinated junctions and glia *(Colleoni M., Sacerdote P. 2010).*

1.2.3 Mechanisms of Neuropathic Pain

Neuropathic pain is associated with pathological changes at many sites along the neural axis. For the spinal nerve ligation model of neuropathic pain, numerous abnormalities that may account for neuropathic pain have been identified in both the peripheral and the central nervous system *(Borzan J., Meyer R.A. 2009).* Spontaneous neural activity and ectopic sensitivity to mechanical stimuli develops at the site of nerve injury. The expression of different molecules in the dorsal root ganglion of the injured nerve is up- or downregulated, reflecting the loss of trophic support from the periphery. Spontaneous neural activity develops in the DRGs. The distal part of the injured nerve undergoes Wallerian degeneration, exposing the surviving nerve fibers from uninjured portions of the nerve to a milieu of cytokines and growth factors. Partial denervation of the peripheral tissues leads to an excess of trophic factors from the partly denervated tissue that can lead to sensitization of primary afferent nociceptors. The expression of different molecules in the dorsal root ganglion of the uninjured nerve is up- or downregulated, reflecting the enhanced trophic support from the periphery. Sensitization of the postsynaptic dorsal horn cell develops, leading to an augmentation of the response to cutaneous stimuli. Activated microglial cells contribute to the development of this dorsal horn sensitization. Changes in descending modulation of dorsal horn neurons also may contribute to the enhanced responsiveness of dorsal horn neurons *(Campbell J.N., Meyer R.A. 2006).*

1.2.3.1 Peripheral Nervous System and Ectopic activity

The importance of the peripheral nervous system in neuropathic pain is supported by the observation that peripherally acting drugs alter neuropathic pain behavior. For example, the cannabinoid receptor 2 (CB2) is selectively expressed in the peripheral nervous system, and selective CB2 agonists were effective in reversing mechanical and heat hyperalgesia in an animal model of neuropathic pain. In addition, peripherally acting opiates have been shown to ameliorate neuropathic pain behavior *(Borzan J., Meyer R.A. 2009).*

An important feature of neuropathic pain is pain in the absence of an identifiable stimulus. Spontaneous pain arises as a result of ectopic action potential generation within the nociceptive pathways and does not originate in peripheral terminals in response to a stimulus. Theoretically, ectopic activity could be generated at any anatomical level proximal to those brain regions that mediate the sensory experience. Compelling evidence for peripheral neuropathic pain, however, points to substantial ectopic activity arising in primary sensory neurons. After peripheral nerve damage, spontaneous activity is generated at multiple sites, including in the neuroma (the site of injury with aborted axon growth), in the cell body of injured dorsal root ganglia (DRG) neurons, and in neighboring intact afferents. Spontaneous pain may arise both from ectopic activity in nociceptors and from low-threshold large myelinated afferents due to central sensitization and altered connectivity in the spinal cord. After spinal cord injury, spontaneous pain may result from increases in the intrinsic excitability of second-order neurons *(Costigan M. et al. 2009).* Under physiological conditions, activation of unmyelinated (C-fibre) and thinly myelinated (Aδ-fi bre) nociceptive afferent fibres indicates potential tissue damage, which is reflected in the high thresholds of nociceptors for mechanical, thermal, and chemical stimuli. These conditions change dramatically in neuropathic pain states. After a peripheral nerve lesion, spontaneous activity is evident in both injured and neighbouring uninjured nociceptive afferents *(Baron R. et al. 2010)* (Fig. 4).

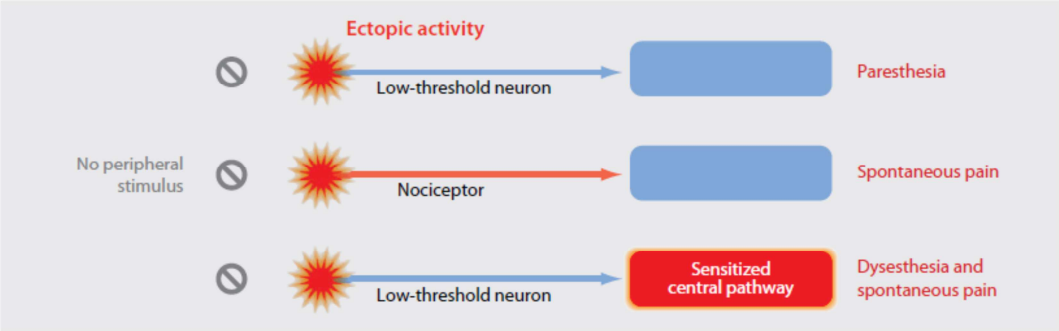

Figure 4 Stimulus-response relations and generation of ectopic impulses *(Costigan M. et al. 2009).*

Voltage-gated sodium channels contribute largely to the generation of ectopic activity as indicated by the robust inhibitory effects of local anesthetics, which are nonselective sodium channel blockers *(Sheets P.L. et al. 2008).* Increasing levels of mRNA for voltage-gated sodium channels seem to correlate with ectopic activity, and increased expression of sodium channels in lesioned and intact fibres might lower action potential threshold until ectopic activity takes place *(Baron R et al. 2010).* DRG neurons express several sodium channels however, which of these channels is responsible for the abnormal generation of action potentials is not entirely clear *(Costigan M. et al. 2009).*

Further evidence for the crucial role of voltage-gated sodium channels in chronic pain states comes from patients with erythromelalgia and paroxysmal extreme pain disorder who have severe ongoing pain at different sites of the body. These hereditary disorders are caused by gain-of-function mutations in the SCN9A gene that encodes α subunit of the Nav1.7 voltage-gated sodium channel *(Hains B.C. et al. 2007).* This channel is expressed predominantly in the small-diameter primary afferents and is believed to participate in the action potential initiation at the peripheral nerve terminals.

Whereas the TTX-resistant, sensory neuronspecific channels, Nav1.8 and Nav1.9, are reportedly downregulated in injured afferents, Nav1.8 is upregulated in the uninjured neighboring fibers *(Borzan J., Meyer R.A. 2009).*

In addition to voltage-gated sodium channels, several other ion channels probably undergo alterations after a nerve lesion, such as voltage-gated potassium channels, calcium channel and hyperpolarization-activated cyclic nucleotide-modulated channel, which might also contribute to changes in membrane excitability of nociceptive nerves.

The hyperpolarization-activated cyclic nucleotide-modulated channel (HCN), which contributes to the pacemaker current l(h), also generates ectopic activity in DRG neurons after nerve injury *(Luo L. et al. 2007)*. Opening the neuronal voltage-gated potassium channel subfamily Q (KCNQ), which is a mediator of the M current, selectively reduces activity in axotomized but not uninjured axons *(Roza C., Lopez-Garcia J.A. 2008)* and in human C-fiber axons *(Lang P.M. et al. 2008)*, suggesting that this channel may be involved in regulating ectopic activity.

Mice with a deletion of Cav2.2 (the N-type calcium channel) show reduced neuropathic pain-like behavior (Saegusa et al. 2001). Intrathecal delivery of ω-conopeptide MVIIA, which blocks Cav2.2 in a non-use-dependent fashion, decreases neuropathic pain in preclinical models and patients, presumably by reducing transmitter release from nociceptors *(McGivern J.C. 2006)*. The calcium channel auxiliary α2δ1 subunit helps stabilize the poreforming α subunit of these channels in the membrane. Gabapentin and pregabalin, among the first-line treatments for neuropathic pain *(Dworkin R.H. et al. 2007)*, bind to the α2δ1 protein, interfere with the interaction between the auxiliary subunit and the α subunit, and impair membrane insertion of the channel *(Hendrich J. et al. 2008)*. Both Cav2.2 and α2δ1 subunits are upregulated in DRG neurons following nerve injury *(McGivern J.C. 2006)*, which suggests that an N-type calcium channel complex may play a dominant role in pathological nociceptive signal transmission from the periphery *(Costigan M. et al. 2009)*.

Moreover, nerve injury also induces upregulation of various receptor proteins such as the transient receptor potential V1 (TRPV1). TRPV1 is located on subtypes of peripheral nocicepive endings and is physiologically activated by noxious heat at about 41°C (*Caterina M.J. et al. 2001*). After a nerve lesion, TRPV1 is downregulated on injured nerve fibres but upregulated on uninjured C-fibres (*Ma W. et al. 2005*). This novel expression of TRPV1 and additional sensitisation to heat by

intracellular signal transduction might lead to spontaneous nerve activity induced by normal body temperature, if the threshold of TRPV1 is reduced to below 38°C (*Bigss J.E. et al. 2008)*. Clinically, patients with such underlying pain mechanisms can also be characterised by the presence of heat hyperalgesia in addition to ongoing burning pain. Similarly, ongoing ectopic discharges of nociceptive afferent fibres have been recently identifyed in a patient with painful neuropathy in combination with cold allodynia. Abnormal responses to cold and topical application of menthol indicated that a nerve lesion triggered abnormal function or expression of TRPM8, a cold-sensitive receptor of the TRP family (*Serra J. et al. 2009*).

1.2.3.2 Low-Threshold Aβ Fiber-Mediated Pain

Neuropathic pain involves a profound switch in sensitivity such that low-intensity input can generate pain, a disruption of the normal pattern of pain specificity *(Perl E.R. 2007)*. The hypersensitivity occurs largely in the absence of peripheral sensitization; includes areas outside of injured nerve territories; is typically associated with a loss of C-fiber peripheral terminals, and sensitivity *(Devigili G. et al. 2008)*; and disappears when conduction in large myelinated fibers is blocked *(Campbell J.N. et al. 1988)*. Furthermore, ablation of the vast majority of nociceptor neurons does not alter the development and manifestation of neuropathic pain *(Abrahamsen B, et al. 2008)*, whereas selective pharmacological blockade of large neurofilament-200-positive Aβ fibers abolishes dynamic tactile allodynia in nerve injury models *(Yamamoto W. et al. 2008)* (Fig. 5). The obvious conclusion from these data is that low-threshold Aβ fibers, which normally signal innocuous sensations, begin after neural lesions to produce pain *(Khan G.M. et al. 2002; Witting N. et al. 2006)*. In keeping with this finding, loss of the PKCγ interneurons in the ventral part of the superficial dorsal horn (lamina III) that are driven only by Aβ fiber innocuous input *(Neumann S. et al. 2008)* leads to reduced neuropathic but preserved nociceptive pain *(Malmberg A.B. et al. 1997)*. Furthermore, after nerve injury polysynaptic and monosynaptic Aβ fiber input to neurons increases in the most superficial laminae of the dorsal horn *(Okamoto M. et al. 2001)*, an area that

normally only receives input from Aδ and C fibers *(Lu Y., Perl E.R. 2005).* Although noxious stimuli activate ERK MAP kinase in superficial dorsal horn neurons in noninjured animals *(Ji R.R. et al. 1999)*, after peripheral nerve injury Aβ fiber stimulation acquires this capacity *(Matsumoto M.et al. 2008)*. Tactile stimulation also begins to induce c-Fos in these nociceptive neurons *(Bester H. et al. 2000).* These structural changes may be an anatomical substrate for the entry of low-threshold Aβ fiber input into nociceptive pathways after nerve injury. Somehow, as a consequence of peripheral nerve injury, low-threshold input from large myelinated fibers is transferred from nonnociceptive to nociceptive circuits in the spinal cord *(Costigan M et al. 2009)*. This means that normally innocuous tactile stimuli such as light brushing or pricking the skin become painful. Similar mechanisms might take place not only within the spinal cord, but also at supraspinal levels, as has been reported in patients with central pain *(Baron R. et al. 2010)*.

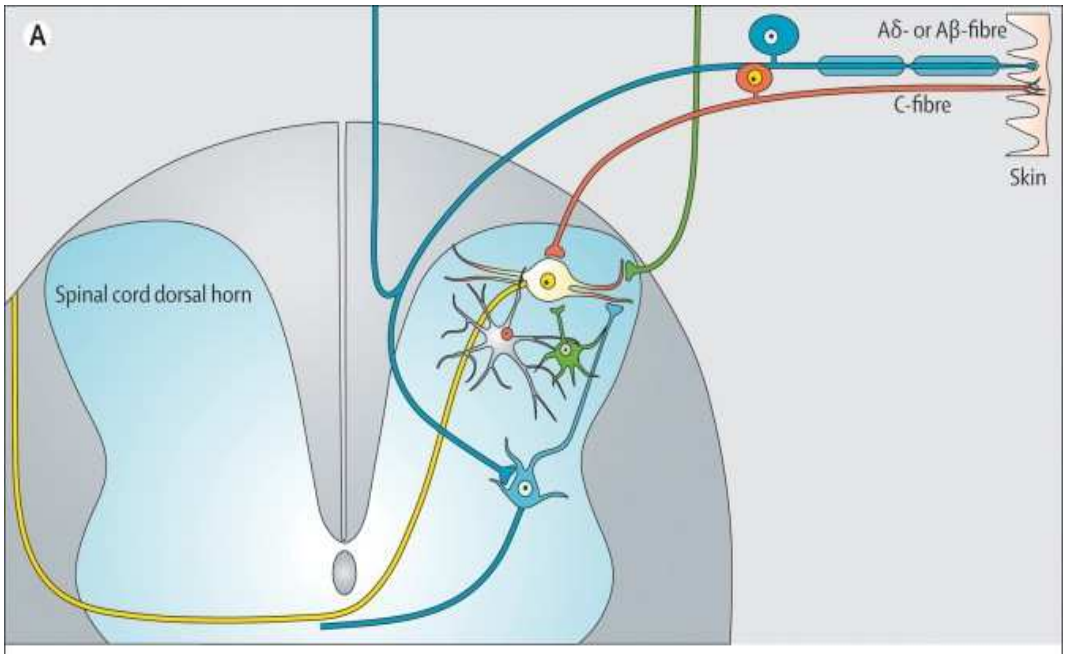

Figure 5 Pathophysiological mechanisms of neuropathic pain

Primary aff erent pathways and their connections in the spinal cord dorsal horn. Note that nociceptive C-fi bres (red) terminate at spinothalamic projection neurons in upper laminae (yellow neuron). Non-nociceptive myelinated A-fi bres project to deeper laminae. The second-order projection neuron is a WDR type—it receives direct synaptic input from nociceptive terminals and also multisynaptic input from myelinated A-fi bres (non noxions information, blue neuron system). Interaction with microglia (grey cell) facilitates synaptic transmission. GABAergic interneurons (green neuron) normally exert inhibitory synaptic input on the WDR neuron. Furthermore, descending modulatory systems synapse at the WDR neuron (only the inhibitory projection, green descending terminal) *(Baron R. et al. 2010)*.

1.2.3.3 Peripheral sensitization

Following many types of tissue injury, including nerve injury, primary afferents develop lower activation threshold, become more responsive to natural stimuli, and develop larger receptive fields. In other words, they become sensitized. A change occurs in the transduction mechanisms that convert external stimuli (heat, touch, etc.) to electrical signals of neurons. After injury, proteins and ion channels in the primary afferents can undergo structural changes (e.g., phosphorylation) or primary afferents can develop altered gene expression that signals the production of new molecules. Consequently, previously nonnoxious stimuli may now be sufficient to activate a given channel or receptor, they may remain active for a longer period of time, or new channels may become activated by different factors within the injury site. These changes are responsible for peripheral sensitization. It is well known that cyclo-oxygenase enzymes play a role in inflammatory pain conditions by converting arachidonic acid into proinflammatory factors such as prostaglandin E2 (PGE2). Prostaglandins have also been shown to contribute to peripheral sensitiza- tion in neuropathic pain by shifting the activation threshold of tetrodotoxin-resistant sodium channels (TTX-R Naþ) Nav1.8 and Nav1.9. PGE2 binds to G-protein-coupled receptors and increases cyclic AMP, which can sensitize TTX-R Naþ channels by activating protein kinases A and C.

Following peripheral nerve lesion, peripheral sensitization occurs in the uninjured fibers innervating the same territory. Sensitization of uninjured fibers occurs partly because Wallerian degeneration of the injured nerve results in a release of a number of proinflammatory factors, including cytokines and growth factors, that can alter the activation threshold of the surviving neurons. These changes drive to sensitivity of uninjured afferents to chemical (TNF-α), mechanical, and heat stimuli following peripheral nerve injury *(Borzan J., Meyer R.A. 2009)*(Fig. 6).

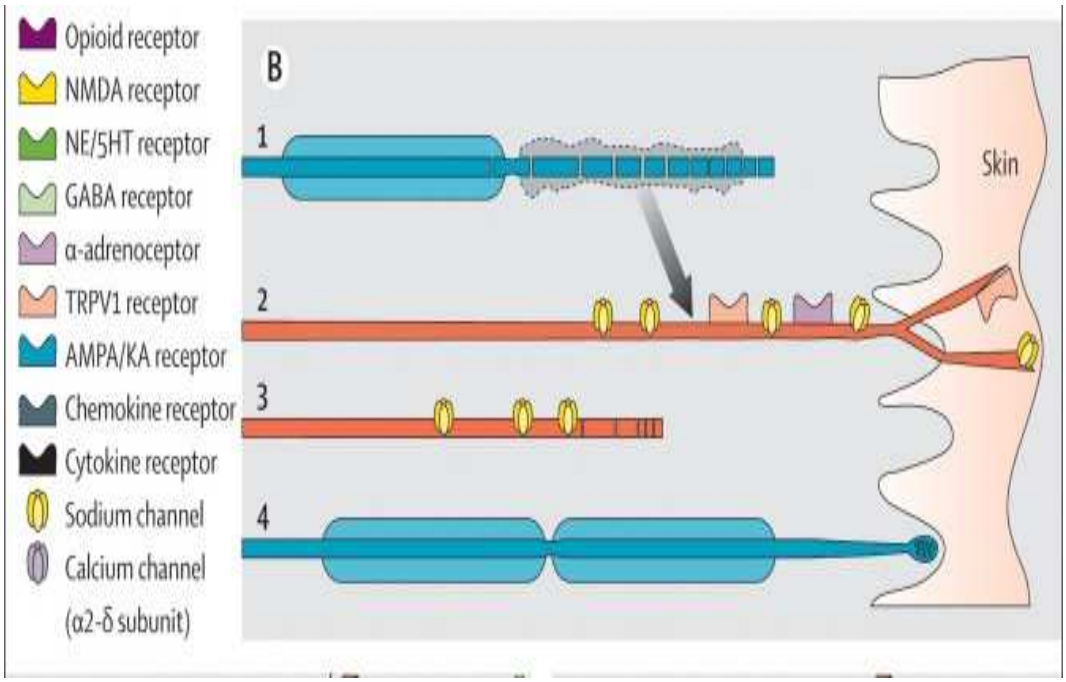

Figure 6 Peripheral changes at primary aff erent neurons after a partial nerve lesion, leading to peripheral sensitisation.

Some axons are damaged and degenerate (axons 1 and 3) and some are still intact and connected to the peripheral end organ (skin; axons 2 and 4). Expression of sodium channels is increased on damaged neurons (axon 3), triggered as a consequence of the lesion. Furthermore, products such as nerve growth factor, associated with Wallerian degeneration and released in the vicinity of spared fi bres (arrow), trigger expression of channels and receptors (eg, sodium channels, TRPV1 receptors, adrenoreceptors) on uninjured fi bres *(Baron R. et al. 2010).*

1.2.3.4 Central nervous system and central sensitization

Peripherally directed treatments do not always help in alleviating neuropathic pain, indicating that changes also occur in the central nervous system. Nerve injury can lead to sensitization of central pain-signaling neu- rons as well as changes in the properties of the inhibi- tory interneurons and descending inhibition *(Borzan J., Meyer R.A. 2009).*

Central sensitisation might develop as a consequence of ectopic activity in primary nociceptive afferent fibres and structural damage within the CNS itself might not be necessarily involved. Ongoing discharges of peripheral afferent fibres that release excitatory aminoacids and neuropeptides within the dorsal horn of the spinal cord lead to pre- and postsynaptic changes and altered response of these central of second neurons *(Baron R. et al. 2010).* Presynaptic functional changes include alterations in the synthesis of transmitters and neuromodulators *(Obata K. et al. 2003)* and in calcium channel density *(Hendrich J. et al. 2008).* Postsynaptic changes involve phosphorylation of N-methyl-D-aspartate (NMDA) subunits and AMPA receptor *(Ultenius C. et al. 2006)* and increased receptor density due to trafficking and enhanced synthesis of ion channels and scaffold proteins *(Cheng H.T. et al. 2008).*

Wind-up is a single synapse phenomenon of enhanced responsiveness of a central neuron following continuous low-frequency nociceptor activation from the periphery. Central sensitization can result from spatial and temporal summation of wind- up. Wind-up is a short-lasting phenomenon (tens of seconds) largely mediated by glutamatergic activation of NMDA receptors. Hence, blocking NMDA recep- tors abolishes wind-up to various stimuli in healthy individuals and reduces neuropathic pain in select con- ditions, such as painful phantom limb and PHN *(Borzan J., Meyer R.A. 2009).*

Drugs that attenuate central sensitization by acting on calcium chan- nel subunits to decrease transmitter release and on NMDA channels to reduce transmitter action are effective treatment options in neuropathic pain *(Costigan M. et al. 2009)* even if secondary hyperalgesia (pain in the area surrounding the injury and attributed

to central sensiti- zation) is generally not altered by inhibiting NMDA receptors *(Borzan J., Meyer R.A. 2009).*

Enhanced responsiveness of dorsal horn neurons after central sensitization lasts for prolonged periods of time, suggesting additional or alternative mechanisms to the wind-up phenomenon. Specifically, the release of substance P from primary afferent neu- rons can facilitate depolarization of dorsal horn neurons by directly activating NK-1 receptors and by subsequent modulation of NMDA receptor activation *(Borzan J., Meyer R.A. 2009).*

Although central sensi- tization was first described in the dorsal horn, similar synaptic changes occur in structures involved in the emotional aspects of pain such as the amygdala, anterior cingulate gyrus, and prefrontal cortex *(Pedersen L.H. et al. 2007),* and these may represent a substrate for long-term cognitive and mood changes that are learned and retained, for example, conditioned fear and addictive behavior.

Central sensitization is different from centralization, which hypothesizes that, after pe- ripheral nerve injury, changes intrinsic to the CNS develop and maintain pain independent of any ongoing peripheral input *(Devor M. 2006)*. These changes potentially include increased ex- citability *(Balasubramanyan S. et al. 2006),* structural alterations in synaptic circuitry *(Woolf C.J. et al. 1992),* degeneration of inhibitory in- terneurons *(Scholz J. et al. 2005),* and alterations in the brain stem regulation of nociceptive transmission *(Vera-Portocarrero L.P. et al. 2006).*

1.2.3.5 Disinhibition

Some of the spinal cord interneurons rich in GABA, glycine, or opioid peptides have an inhibitory influ- ence on synaptic transmission *(Borzan J., Meyer R.A. 2009).* Pharmacological removal of GABAergic or glycinergic control provokes tactile allodynia and increases synaptic currents from Aβ fibers to nociceptive lamina I neurons *(Costigan M. et a. 2009)*. Peripheral nerve injury can lead to impaired tonic inhibitory function of these interneurons by reducing the synthesis or release of inhibitory neurotransmitters or to apoptosis of these interneurons due to glutamate neurotoxicity *(Borzan J., Meyer R.A. 2009).*

Descending pathways that modulate the spinal transmission of nociceptive input orig- inate in the anterior cingulate gyrus, amyg- dala, and hypothalamus and are relayed to the spinal cord through brain stem nuclei in the periaqueductal gray and rostroven- tral medulla. The inhibitory transmitters in these pathways include norepinephrine, 5-hydroxytryptamine, and endogenous opioids. After nerve injury, this intricate system of inhibitory control shifts. Tonic noradrenergic inhibition that acts on α2-adrenoceptors appears to be suspended *(Rahman W. et al. 2008),* and the net effect of descending serotoniner- gic input changes from inhibition to facilitation *(Bee L.A., Dickenson A.H. 2008, Vera-Portocarrero L.P. et al. 2006).* Amine uptake inhibitors like tricylic antidepressants or serotonin nore- pinephrine reuptake inhibitors (SNRIs) boost endogenous inhibition by increasing the levels of norepinephrine *(Matsuzawa-Yanagida K. et al. 2008).*

Some studies indicate that endogenous opioids serve an anti- nociceptive role following nerve injury. Mice lacking the m opioid receptors show enhanced nerve injury-induced behavioral hyperalgesia and wind-up compared to wild-type animals. Intrathecal administration of delta opioid ago- nist reversed mechanical and cold hyperalgesia following spinal nerve ligation *(Borzan J., Meyer R.A. 2009).*Following nerve injury, primary afferents reduce their expression of μ opioid receptors, and dorsal horn neurons are less sensitive to inhibition by μ opioid agonists *(Kohno T. et al. 2005).*

Furthermore, several different mechanisms contribute to a loss of pre- and postsynaptic GABAergic inhibition in the spinal cord. In nociceptive lamina I neurons, the transmem- brane gradient for chloride ions changes after nerve injury so that activation of $GABA_A$ receptors no longer leads to hyperpolarization. Instead, it may induce depolarization, provoking paradoxical excitation and spontaneous activity *(Keller A.F. et al. 2007).* BDNF released from active microglia causes this disturbance by inducing a downregulation of the potassium chloride cotransporter isoform 2 *(Coull J.A. et al. 2005)* (Fig. 7).

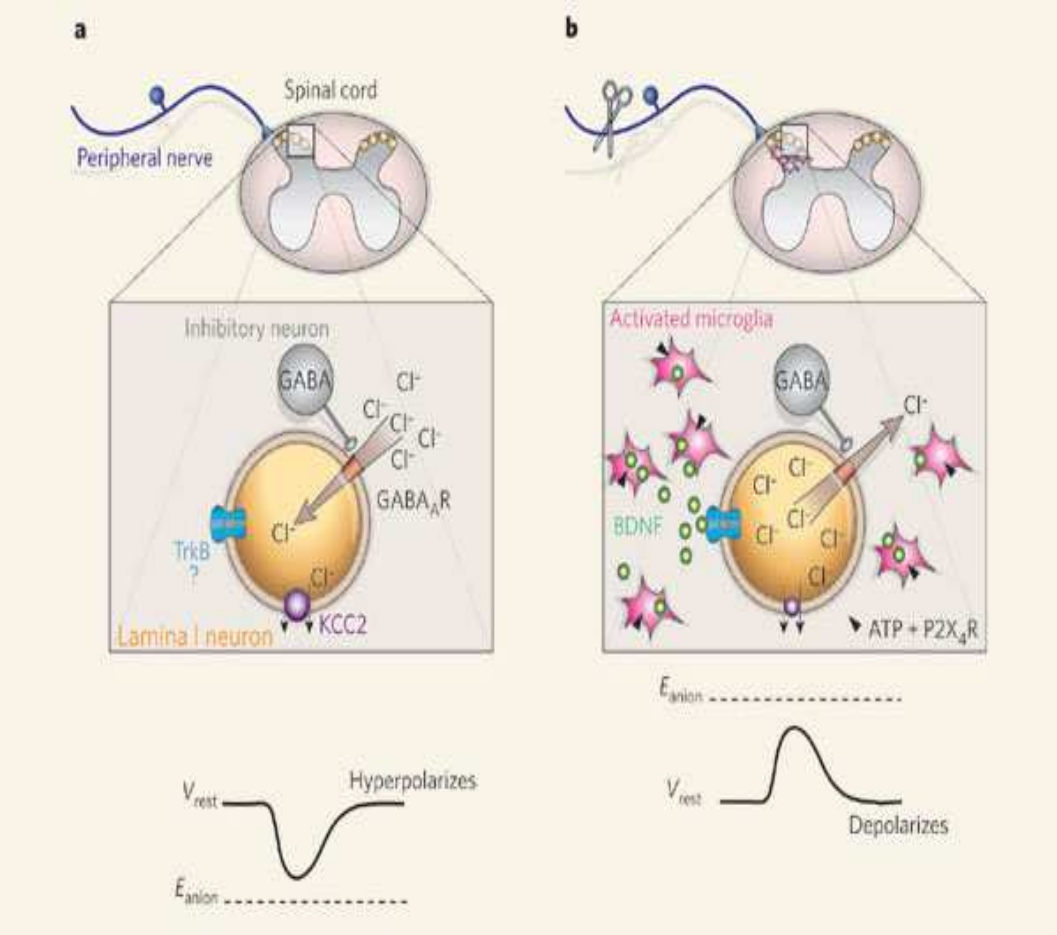

Figure 7 A pathway of pain. Microglial neuronal signaling mediated by BDNF disrupts inhibition of rat lamina I spinal-cord neurons and maintains neuropathic pain.

a) Activation of $GABA_A$ receptors ($GABA_AR$) normally leads to an influx of anions (principally chloride, Cl^-), causing hyperpolarization (inhibition), because the potential at which the anion flux switches from inward to outward (E_{anion}) is negative with respect to the resting membrane potential of the neuron (V_{rest}).

b) Following peripheral nerve injury, activated microglia (with ATP-stimulated P2X4 receptors) release BDNF, which acts on the TrkB receptor to modify E_{anion}, probably by reducing levels of the potassium-chloride co-transporter KCC2. As Eanion is now positive with respect to V_{rest}, $GABA_A$-receptor activation leads to an efflux of anions, depolarizing the lamina I neurons. Blockade of this microglial–neuronal signalling pathway alleviates chronic neuropathic pain in the rat model *(Torsney C., Macdermott A.B. 2005).*

Independently, inhibition in the superficial dorsal horn of the spinal cord is compromised by a loss of GABAergic interneurons *(Scholz J. et al. 2005)*, reducing afferent stimulation-evoked GABAergic inhibitory postsynaptic currents (IPSCs) *(Moore K.A. et al. 2002)*. Preventing apoptotic cell death fully restores GABAergic IPSCs and attenuates mechanical allodynia, hyperalgesia, and cold allodynia after nerve injury *(Scholz J. et al. 2005)*. Loss of spinal inhibitory interneurons may contribute to the persistence of neuropathic pain, although pain-like behavior after sciatic nerve injury in the absence of neuronal cell death has been reported *(Polgar E. et al. 2005)*. Despite the apparent role of GABAergic disinhibition in neuropathic pain, $GABA_A$ receptor modulators such as benzodiazepines or $GABA_B$ receptor agonists are rarely used to treat neuropathic pain because they have a narrow therapeutic window. Specific GABA agonists that bind to the α2 or α3 but not α1 subunits of spinal $GABA_A$ receptors may allow analgesia without sedation or motor impairment *(Knabl J. et al. 2008)*.

1.2.3.6 Neurodegenerazione

Both primary sensory and dorsal horn neurons die after peripheral nerve injury. Primary afferents degenerate after transection of their peripheral axons, with a much greater loss of small-diameter neurons, including nociceptors, than large myelinated neurons *(Okamoto M. et al. 2001, Tandrup T. et al. 2000)*. A loss of ~20% of superficial dorsal horn neurons occurs after partial peripheral nerve injury. The degeneration of spinal neurons occurs protracted over several weeks and is most likely a consequence of sustained ectopic activity of primary sensory afferents and glutamate-mediated excitotoxicity *(Scholz J. et al. 2005)*. Magnetic resonance imaging (MRI) investigations in patients with chronic neuropathic pain hint that neurodegeneration may also occur in the brain. Voxel-based morphometry shows decreases in gray matter volume and density in MRIs of the brain of patients with chronic back pain, phantom pain, migraine, tension-type headache, and fibromyalgia, although with varying degree and regional distribution *(May A. 2008)*. The nature of these structural changes remains to be determined, as well as whether

neurodegeneration is a cause and if analgesic treatment can prevent the changes. These findings raise the possibility that neuropathic pain may have elements that resemble neurodegenerative diseases and requires neuroprotective treatment strategies *(Costigan M. et al. 2009).*

1.2.3.7 Role of the Sympathetic Nervous System

In some patients, blockade of the sympathetic nervous system leads to pain relief. This sympathetically maintained pain (SMP) is thought to depend on the peripheral release of norepinephrine from sympathetic efferent fibers that activates and sensitizes primary afferents. Animal studies have shown that peripheral nerve injury can result in de novo expression of a1 adrenergic receptors in adjacent uninjured afferents and sensitization to adrenergic agonists, indicating that the coupling site between the sympathetic and pain systems may be in the primary afferent fibers. In human studies, intradermal norepinephrine injections caused pain in patients with SMP but not in healthy controls. Some patients with complex regional pain syndrome have SMP and gain pain relief following sympathetic ganglion block or after intravenous administration of phentolamine, an a-adrenergic antagonist. The sympathetic nervous system has been implicated in central pain mechanisms as well. Neuropathic pain patients with strokeinduced central pain frequently complain of exacerbated pain under stress and have areas of abnormal blood flow and sweating *(Borzan J., Meyer R.A. 2009).*

1.2.3.8 Influence of the Immune system

In addition to the obvious role of the immune system in inflammatory pain, there is accumulating evidence that immune factors are of importance in neuropathic pain *(Borzan J., Meyer R.A. 2009).*

In the PNS, immune surveillance is performed by macrophages, which identify and clear cellular debris and present surface antigens to activate T-

lymphoctyes. Both macrophages and T-lymphocytes communicate via cytokines and chemokines with neurons, Schwann cells, and DRG satellite cells. Macrophage activation is a central component of the Wallerian degeneration distal to axonal injury, and immune activation in the injured nerve and DRG appears to contribute to pain hypersensitivity *(Scholz J., Woolf C.J. 2007).*

Damaged or denervated Schwann cells secrete leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1). Upregulation of LIF following injury results in increased release of substance P and other molecules that contribute to nociceptor sensitization. MCP-1 is a chemokine cytokine that binds to chemokine receptor CCR2 and has been shown to activate monocytes and depolarize nocicep- tors following nerve injury either directly or by promoting the release of proinflammatory factors from surrounding nonneuronal cells. Cytokine IL-1β promotes expression and release of NGF that can lead to sensitization of primary afferents. Deletion of IL-1β or overexpression of IL-1βantagonist largely diminished the development of neuropathic pain following spinal nerve ligation or axotomy. These results complement previous evidence that injection of IL-1β or TNF-α in the sciatic nerve causes mechanical and heat hyperalge- sia *(Borzan J., Meyer R.A. 2009).*

Microglia, the macrophages of the CNS, are massively activated in the dorsal horn soon after peripheral nerve injury. Microglial activation occurs in a topographically organized fashion close to the central terminals of injured afferents *(Beggs S., Salter M.W. 2007, Scholz J. et al. 2008),* and microglial cells release many immune modulators that contribute to the induction and maintenance of neuropathic pain by altering neuronal function *(Saab C.Y. et al. 2008, Suter M.R. et al. 2007)* For example, painful peripheral neuropathy due to HIV infection may also be caused in part by spinal cord microglia activation. Various approaches to inhibit microglial activation provide direct support that immune cell activation in the central nervous system contributes to neuropathic pain, especially in its development *(Borzan J., Meyer R.A. 2009).*

Immune cells may influence neuronal activation and sensitization through upregulation of purinergic receptors and chemokines, activation of various kinases, and release of various cytokines. Microglia express a number of purinergic receptors.

Two have been demonstrated as important in neuropathic pain: P2X4 and P2X7; these receptor, activated through ATP *(Inoue K. 2006)* leads to BDNF release and IL1β synthesis, respectively. Inhibition of P2X4 after nerve injury alleviates mechanical allodynia and prevents upregulation of P2X4 in the dorsal horn. Genetic deletion of the P2X7 receptor resulted in decreased neuropathic pain, but it is not clear whether this effect is mediated specifically by microglia or includes involvement of other central or peripheral cell types *(Borzan J., Meyer R.A. 2009).*

Fractalkine (CX3CL1), for example, is expressed by neurons and astrocytes, whereas its receptor CX3CR3 is expressed on microglia, suggesting a signaling role by the chemokine between these cells *(Milligan F.D. et al. 2008).* CCL2 (MCP-1) and its receptor CCR2 are both up-regulated in the DRG and distributed to the spinal cord after nerve injury *(White F.A. et al. 2007).* Intrathecal administration of CX3CL1 or CCL2 produces pain in näıve animals, while their neutralization prevents neuropathic pain hypersensitivity *(Abbadie C.2005, Watkins L.H. et al. 2007).*

Neuronal release of chemokine fractalkine activates spinal microglia via CX3CR1 receptor and causes activation of the MAPK p38 pathway. Nerve injury also leads to activation of Src kinase in microglia but not neurons or astrocytes. Spinal inhibition of Src kinase attenuates mechanical allodynia by inhibiting extracellular signal-regulated protein kinase. Increased levels of proinflammatory cytokines have been observed in the spinal cord tissue following peripheral nerve injury. Additionally, intrathecal delivery of cytokine inhibitors can lead to the reduction of neuropathic pain *(Borzan J., Meyer R.A. 2009).*

Another example for neuronglia interactions contributing to neuropathic pain are the matrix metalloproteinases MMP2 and MMP9. They are produced by both neurons and glia and mediate pain hypersensitivity by initiating IL1β cleavage and microglial and astrocytic activation *(Kawasaki Y. et al. 2008).* Inhibition of immune function represents a major avenue for therapeutic intervention for neuropathic pain (Fig. 8)

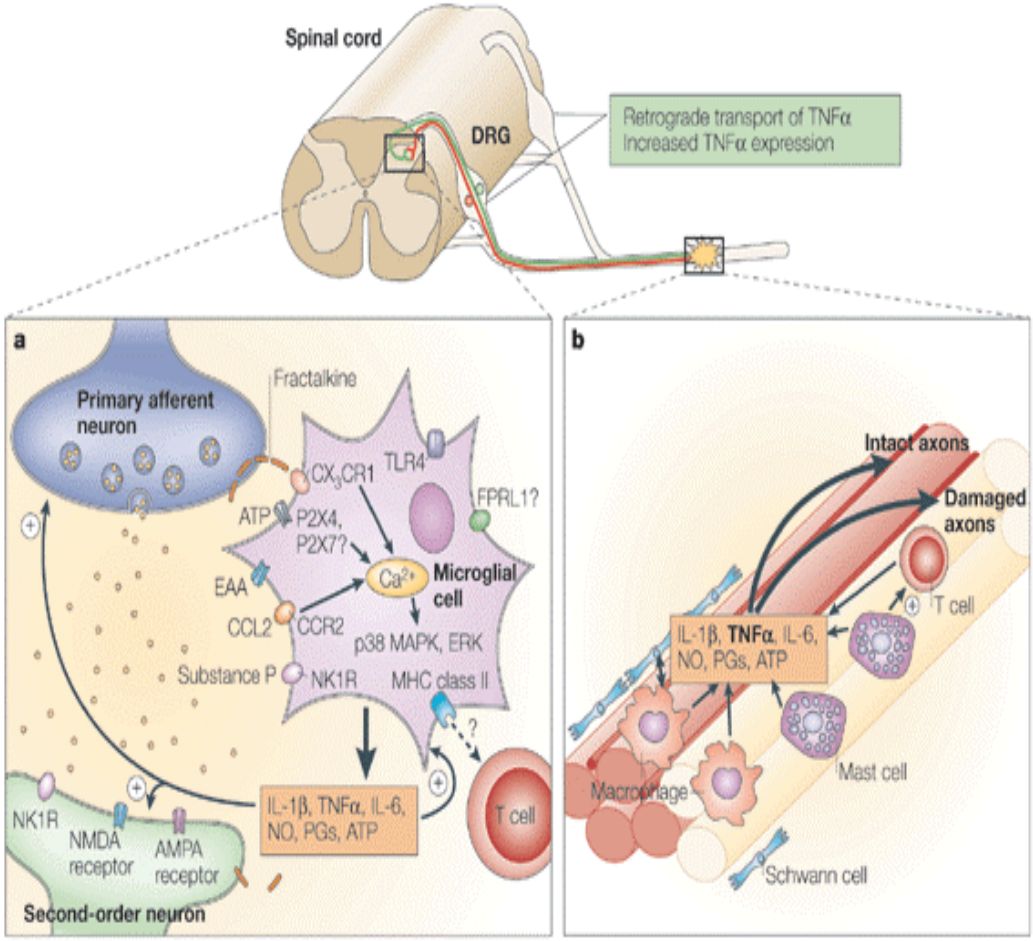

Figure 8 Immune cells present or recruited in the central (a) and peripheral (b) nervous system following nerve injury influence the excitability of primary afferents and second-order neurons.

TNF-α, tumor necrosis factor-a; IL-1b, interleukin-1b; IL-6, interleukin-6; NO, nitric oxide; PGs, ATP and prostaglandins; AMPA, a amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CCR2, CCL2 receptor (CX3CR1), fractalkine receptor; EAA, excitatory amino acids; ERK, extracellular signal-regulated kinase; FPRL1, formyl peptide receptor-like 1; MHC, major histocompatibility complex; NGF, nerve growth factor; NK1R, neurokinin-1 receptor; NMDA, N methyl-Daspartate; P2X4 and P2X7, ionotropic purinoceptors; p38 MAPK, p38 mitogen-activated protein kinase; TLR4, toll-like receptor 4; CCL2, chemokine (C–C motif) ligand 2. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Marchand F, Perretti M, and McMahon SB (2005) Role of the immune system in chronic pain. Nature Reviews Neuroscience 6: 521–532), copyright 2005.

1.2.4 Affective component of neuropathic pain

Pain is a complex sensory and emotional experience that can vary widely between people and even within an individual depending on the context and meaning of the pain and the psychological state of the person. Cognitive and emotional factors have a surprisingly important influence on pain perception. A negative expectation can completely reverse the analgesic effect of a clinical dose of the opioid agonist remifentanil *(Bingel U. et al 2001)*, whereas the expectation of pain relief is an important component of placebo analgesia *(Benedetti F. et al. 2005*). Our emotional state has an enormous influence on pain; a negative emotional state increases pain, whereas a positive state lowers pain *(Villemure C., Bushnell, M.C. 2009).*

Imaging studies show that psychological factors activate intrinsic modulatory systems in the brain, including those involved in opioid-related pain relief. Furthermore, multiple descending pain modulatory systems are implicated in pain relief, with attentional states and emotions activating different systems in the brain. In parallel with increased understanding of the circuitry underlying the psychological modulation of pain, recent evidence shows that chronic pain can lead to anatomical and functional alterations in this same circuitry, resulting not only in pain but also in altered cognition and affect. The wealth of new studies showing alterations in the brains of patients with chronic pain can now be integrated with our increased understanding of the brain circuitry involved in the psychological modulation of pain, allowing us to hypothesize a negative-feedback loop between impaired pain modulatory circuitry and pain processing, leading not only to increased chronic pain but also to cognitive and emotional deficits that are comorbid with the pain (Fig. 9) *(Bushnell M.C. et al. 2013).*

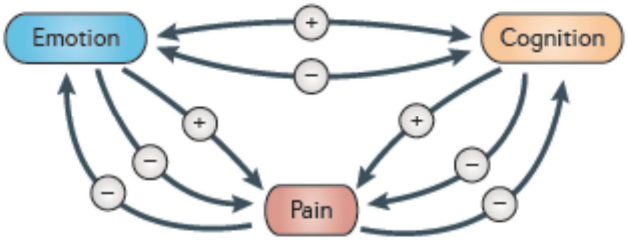

Figure 9 Feedback loops between pain, emotions and cognition. Pain can have a negative effect on emotions and on cognitive function. Conversely, a negative emotional state can lead to increased pain, whereas a positive state can reduce pain. Similarly, cognitive states such as attention and memory can either increase or decrease pain. Of course, emotions and cognition can also reciprocally interact. The minus sign refers to a negative effect and the plus sign refers to a positive effect *(Bushnell M.C. et al. 2013).*

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Neuroimaging studies have now identified several cortical regions in humans that are considered to be important for the perception of pain. The primary and secondary somatosensory cortices (insular and anterior cingulate) and the prefrontal cortices (PFCx) are commonly activated, often bilaterally, and during painful experiences. Furthermore, altered activity in subcortical areas (e.g. brainstem periaqueductal grey (PAG), hypothalamus, amygdala, hippocampus, and cerebellum) is also observed during pain. Thus, activity within several diverse regions of the brain seem to be necessary for the multidimensional experience that is pain *(Apkarian A.V. et al 2009, Apkarian A.V. et al 2005).* Some of these regions comprise the so-called 'pain neuromatrix'*(Melzack R. 2001),* in which multiple inputs are processed to produce an output (neurosignature) that is bespoke for an individual depending on context, mood and cognitive state (*Tracey I., Mantyh P.W. 2007)* Hence, pain is the product of a widely distributed and variably accessible neural network in the brain, rather than an inevitable consequence of noxious stimulation.

Perception is singular psychological construct, with a complex physiological phenomenon in the brain, as that of consciousness. The mode of pain perception can be defined in three distinct conceptual stages related to different functional neuraxis areas: (1) sensory transmission at the brainstem area, also acting in descending

regulation, and the thalamic relay nuclei, while sensory discrimination of spatial/temporal features in primary somatosensory cortex; (2) affect transaction at the secondary somatosensory cortex, insular cortex, amygdala and hippocampus cortices for aversive learning, visceral nociception, and autonomic effect; (3) cognitive attention at the anterior cingukate cortex, prefrontal cortex, as well in response selection or action planning. Transmission of sensory nociception is largely considered as serial processing from brainstem, thalamus , primary somatosensory cortex and secondary somatosensory cortex. This sensory-perceptual system participates in the cerebral functions of detection, localization, timing, learning, relay, integration, ascending transmission and descending regulation of pain. The transaction of painful emotion is probably considered as parallel processing at sites of insular cortex , amygdala , hippocampus , hypothalamus and cingular cortex . This affective-motivational system participates in the functions of affective reaction, visceral activation, multi-sensory integration, homeostasis regulation, fear and memory. Translation of painful cognition is assumed to be carried out by the integration of several cortical areas in prefrontal cortex, primary motor cortex, supplementary motor cortex and posterior parietal cortex. This cognitive evaluation system participates in the functions of executive control, coordination of action/intention and spatial/bodily attention *(Chen A.C. 2008).*

Neuropathic pain has been described as the “most terrible of all tortureswhich a nerve wound may inflict” *(Jaggi A.S., Singh N. 2001).*

Chronic pain induces mood disorders, including depression and anxiety. In addition, the adverseness of pain is amplified or reduced depending on the emotional environment, and conditions of increased anxiety and depression are usually associated with decreased pain tolerance. This vicious circle may trigger, or even result from, neuronal changes *(Goncalves L. et al. 2008).*

Different work in both human patients and laboratory animals has demonstrated that the presence of chronic neuropathic pain causes gross reorganization and functional changes in both cortical and subcortical structures, including the medial prefrontal cortex *(Baliki M.N. et al. 2006, 2008; Metz A.E. et al. 2009),* thalamus *(Apkarian A.V. et al. 2004a),* amygdala *(Han V., Neugebauer J.S. 2005; Ji G. et al., 2010),* and anterior cingulate cortex *(Li X.Y. et al. 2010).* These

areas are associated with functions including learning, memory, fear, and emotional responses and map on to cognitive and emotional problems suffered by chronic pain patients, such as elevated anxiety and depression *(Gore M. et al. 2011)*, problems in emotional decision making *(Apkarian A.V. et al. 2004b)*, working memory *(Dick B.D., Rashiq S. 2007)*, and difficulty in classical conditioning tasks *(Flor H. et al. 2002)(Mutso A.A. et al. 2012)*.

1.3 Endogenous Opioid System

Opioid receptors and their endogenous peptide ligands are largely distributed through the CNS and peripheral tissues. This wide distribution is related to the important role that the opioid system plays in the control of several physiological responses including nociception, emotional behavior, learning and memory and regulation of reward circuits.

The existence of membrane receptors in the brain for opiate drugs was shown for the first time in 1973 by three independent groups *(Pert C.B., Snyder S.H. 1973; Simon E.J. et al. 1973; Terenius L. 1973)*. A few years later, different opioid binding sites were identified, confirming that opioid receptors did not constitute a homogenous population *(Lord J.A. et al. 1977; Martin W.R. et al. 1976)*. The knowledge of the Endogenous Opioid System was largely improved since the molecular characterization and cloning in the mid 1990s of the different opioid receptors μ, δ and κ *(Chen Y. et al. 1993; Evans C.J. et al. 1992; Kieffer B.L. et al., 1992; Meng F. et al. 1993; Thompson R.C. et al. 1993; Yasuda K. et al. 1993)*.

In addition to the well-established three types of opioid receptors, an orphan opioid-like receptor (ORL1) was cloned in 1994. This receptor has nearly 70% sequence homology with the opioid receptors. The endogenous ligand for the ORL1 receptor was isolated and named nociceptin or orphanin FQ *(Meunier J.C. 1995; Reinscheid R.K. et al. 1995)*.

1.3.1 Opioid Peptides

Three families of endogenous peptides enkephalins, dynorphins and β-endorphin derive from proenkephalin (PENK), prodynorphin (PDYN) and proopiomelanocortin (POMC), respectively. PENK is the source of [Met5]- and [Leu5]-enkephalins and several longer peptides. Endogenous opioid peptides such as dynorphin A, dynorphin B and α- and β-neoendorphin and several larger molecules can be generated from PDYN. POMC is the precursor of β-endorphin, α-endorphin and several non opioid peptides. The endogenous opioid peptide-containing neurons have been found in the regions involved in the nociceptive response, e.g. the thalamus, periaqueductal grey, limbic system, cortex and in the spinal cord. Similarly, the autonomic nervous system centers have been shown to be innervated by central and peripheral opioidergic neurons *(Przewłocki R., Przewłocka B. 2001).* A novel group of peptides has been discovered in the brain and named endomorphins, endomorphin-1 (Tyr–Pro–Trp–Phe–NH2) and endomorphin-2 (Tyr–Pro–Phe–Phe–NH2). They are unique in comparison with other opioid peptides, having a characteristic atypical structure and high selectivity towards the μ-opioid receptor *(Zadina J.E. et al. 1997).* Anatomical studies demonstrated a distinct anatomical distribution of endomorphins (e.g. endomorphin-1 is present mainly in the brain and endomorphin-2 in the spinal cord) and their synthesis in separate cellular systems. Another system which has joined the endogenous opioid peptide family is the pronociceptin system comprising the peptides derived from this prohormone, acting at ORL1 receptors *(Meunier J.C. 1995; Reinscheid R.K. et al. 1995)* (Fig.10).

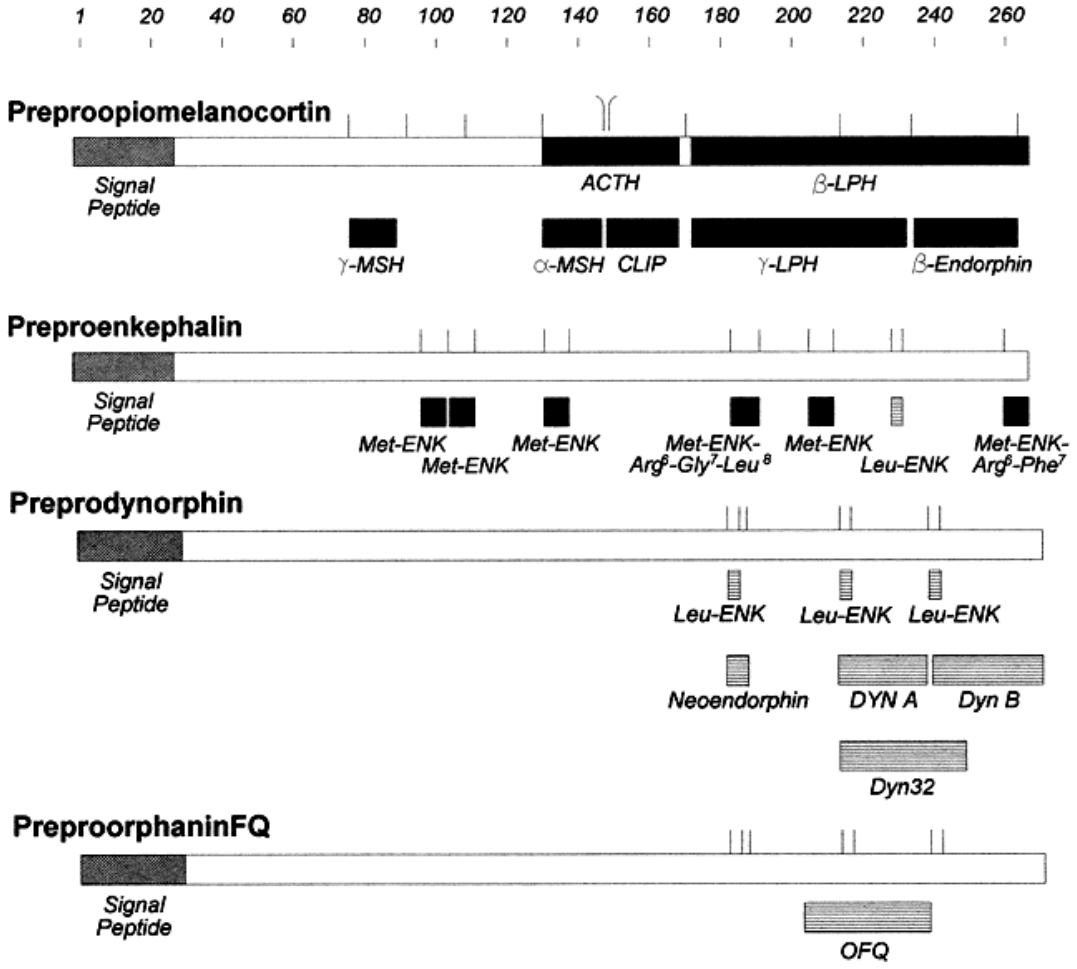


Figure 10 Proopiomelanocortin, preproenkephalin A, preproenkephalin B, and preproorphanin FQ (prepronociceptin) precursor molecules and their related cleavage products: ACTH, β-lipotropin (B-LPH), α-melanocyte-stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP), γ-LPH, β-endorphin, met-enkephalin, leu-enkephalin, dynorphin A, neoendorphin, and orphanin FQ (nociceptin) *(James H. Liu and John L. Mershon, Neurosecretory Peptides).*

1.3.2 Opioid Receptors

Opioid receptors belong to the superfamily of seven transmembrane domain receptors and produce their cellular effects via coupling with toxin-sensitive GTP-binding proteins Gi/Go (Fig 11). Activation of opioid receptors leads to inhibition of cAMP production and voltage gated Ca2+ channels, as well as the stimulation of inwardly rectifying K+ channels and activation of the MAP kinase pathway. At cellular level, all these actions induce inhibition of neuronal activity and a reduction in neurotransmitter release *(Law P.Y. et al. 2000).*

The cloned μ-opioid receptor is a morphine-like receptor, and endomorphins may be its endogenous ligands. The enkephalins bind to the δ-opioid receptor with great affinity, and therefore, are considered to be endogenous δ-opioid receptor agonists. The affinity of β-endorphin binding to μ- and δ-opioid receptors was found to be similar. Dynorphins bind to k-opioid receptors and therefore appear to function as its endogenous ligands. Opioid peptides do not bind exclusively to one specific receptor type but have some affinity for other opioid receptors as well *(Przewłocki R., Przewłocka B. 2001)*. Several subtypes of the opioid receptors (μ1, μ2; δ1 δ2; κ1, κ2, κ3) have been postulated on the basis of pharmacological studies. Cloning of all opioid receptors to date yielded a single receptor type, and the suggested subtypes are possibly alternative splicing products. In addition, oligomerization of various opioid receptors generates unique functional properties *(Jordan B.A.,Devi L.A. 1999).*

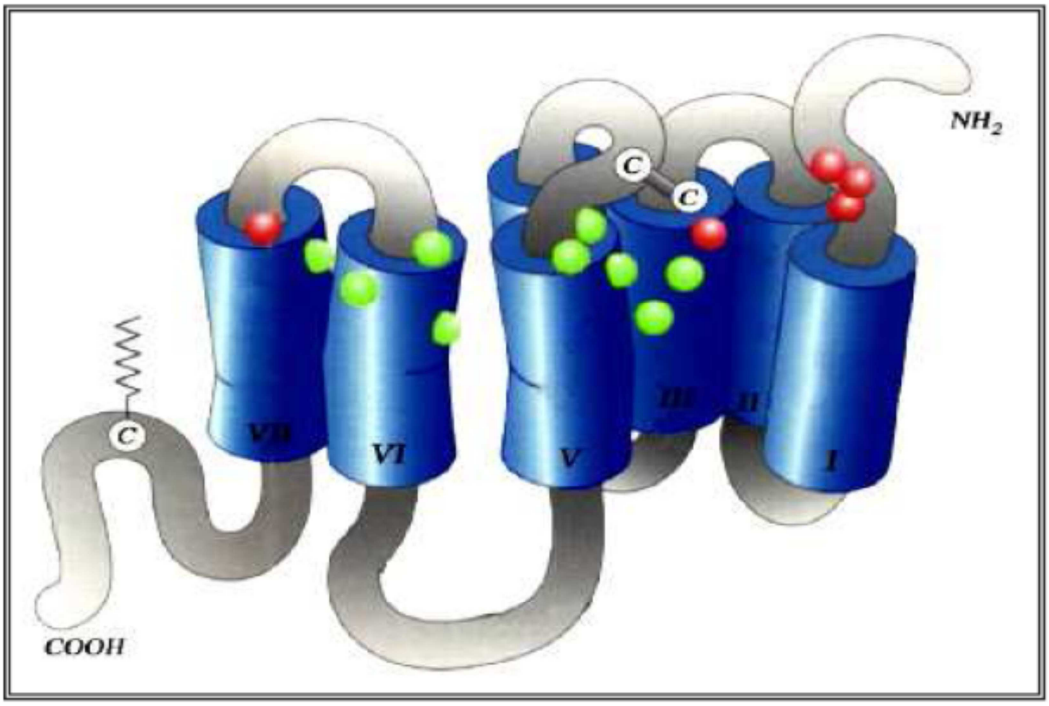


Figure 11 Representation of the opioid receptor structure, constituted by seven transmembrane domains, an extracellular site for interaction with the ligand and an intracellular site for interaction with the G protein of inhibitory (G i / o).

1.3.3 Nociceptin – NOPr system

1.3.3.1 NOP receptor

The NOP receptor was first cloned as an orphan opioid receptor-like receptor from human *(Mollereau C. et al. 1994)* rat *(Bunzow J.R. et al. 1994)* , and mouse *(Nishi M. et al. 1994)* brain, and human lymphocytes *(Halford W.P. et al. 1995; Wick M.J. et al. 1994)*. NOP is a typical G-protein-coupled receptor with seven predicted transmembrane domains (Fig. 12).

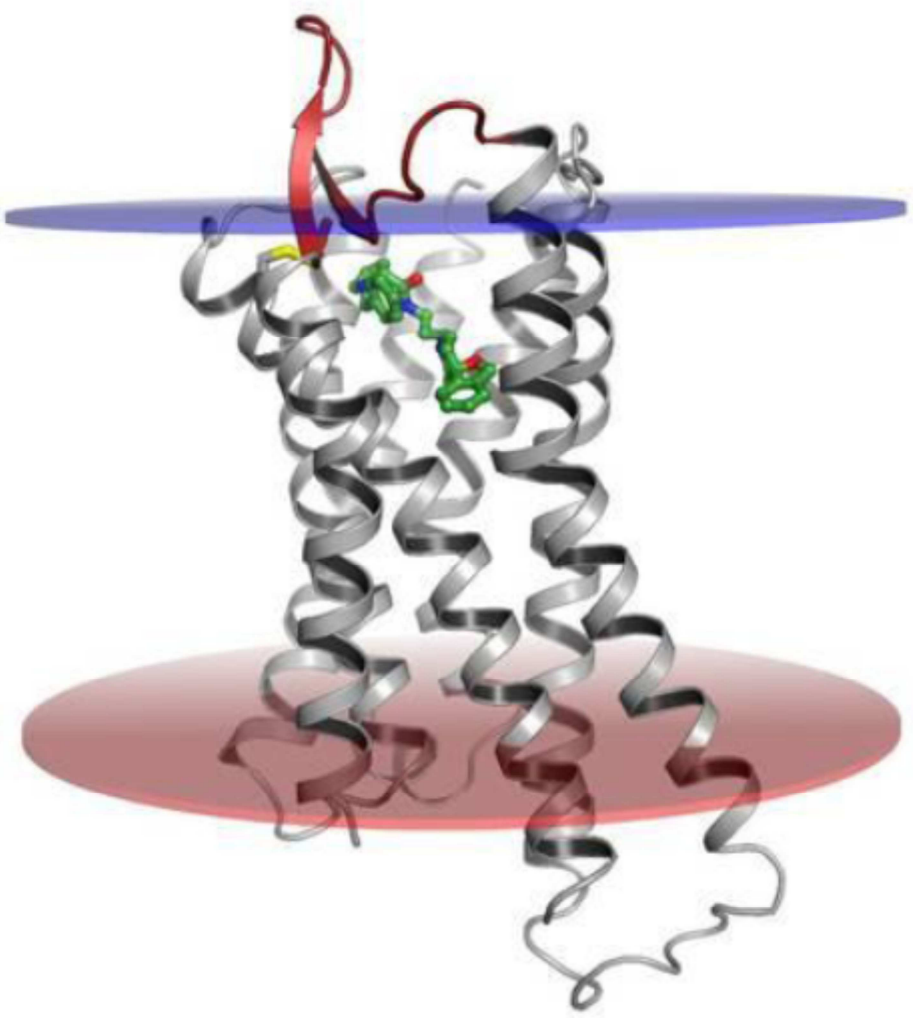


Figure 12 The NOP receptor.

Alignment of the cDNA-deduced amino acid sequences of the human NOP, δ-, μ-, and κ-opioid receptors reveals conserved regions, notably in the transmembrane helices and cytoplasmic loops (Fig. 13). Sequence conservation among the four receptors is highest (>70%) in the second, third, and seventh transmembrane helices, and approximately 50% in the first, fifth, and sixth, but significantly lower (24%) in the fourth. Amino acid sequence identity is also high (56–86%) in the four intracellular loops. All other regions, the C-terminal half of the first exofacial loop excepted, display little amino acid sequence conservation. While the e2 loops of the ORL1, m-, and d-opioid receptors are all of the same length, the highly acidic content of the ORL1 e2 loop is retained only in the k-opioid receptor loop, which contains two insertion sites and is three residues longer. It is also apparent that the charge distributions as a linear function of residue position in the k-opioid and ORL1 receptor e2 loop sequences are quite dissimilar *(Meunier J.C. et al. 2000).* Despite their close homology to the other opioid receptors, only few opioids bind these novel clones, and their affinities are markedly lower than those seen with the other opioid receptors.

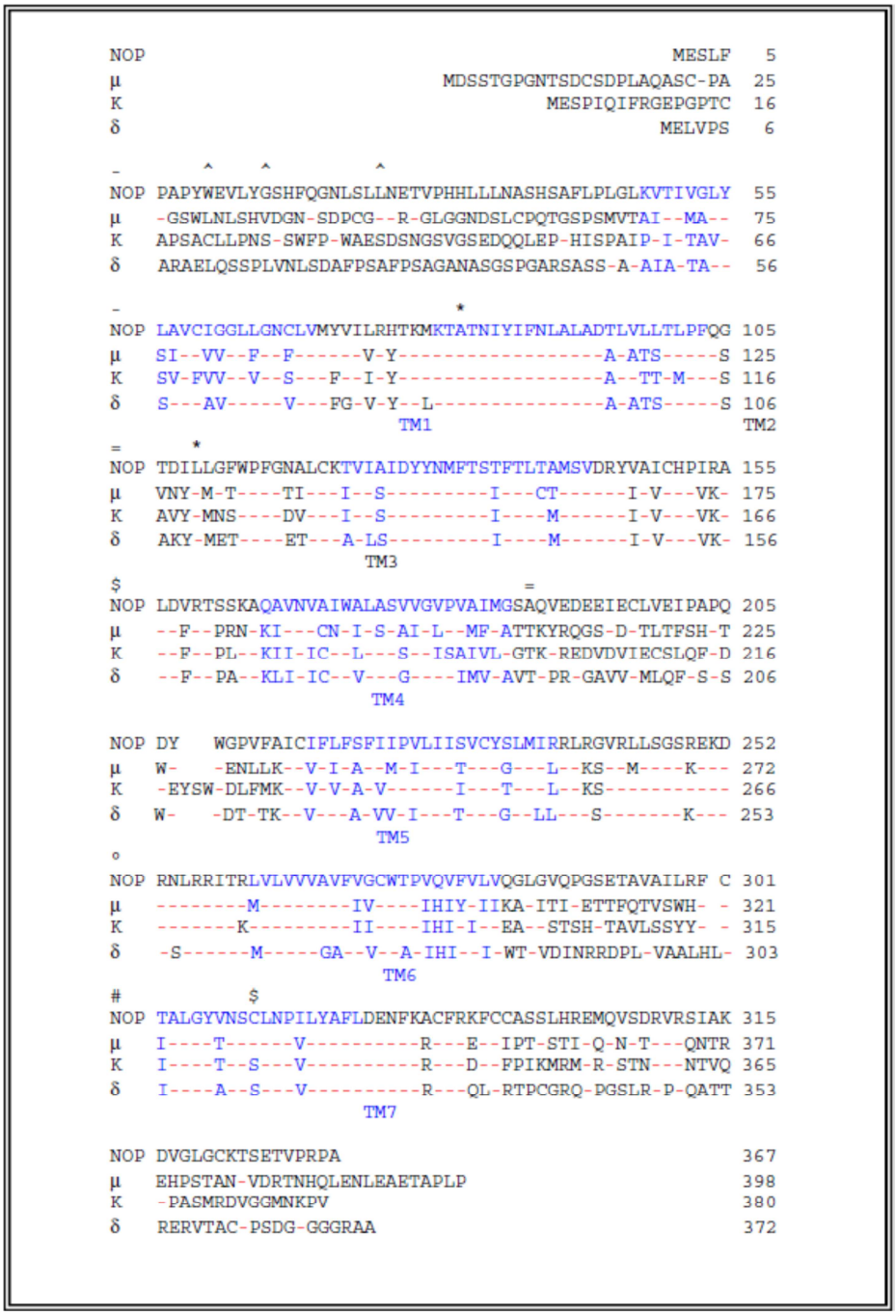


Figure 13 Sequence alignment of the putative opioid receptor with three other opioid receptors. The ammo acids identical to the putative opioid receptor are marked by a dash (-). The seven putative hydrophobic transmembrane domains are underlined and labeled from TM1 to TM7. Gaps are introduced for alignment. Other symbols: (=), the conserved cysteine residues possibly involved with disulfide bond formation; (*), the conserved aspartic acid residues that may interact with the primary amine group found in ligands; (^), the potential N-linked glycosylation sites; (S), the putative protein kinase C sites; (o), the potential CAMP dependent protein kinase site; (#), the potential pahnitoylation site *(Chen Y. et al. 1994)*.

The receptor has three coding exons, similar to the other opioid receptors. The first coding exon yields the amino terminus and the first transmembrane domain. The second coding exon is responsible for the next three transmembrane domains. The splice site between the second and third coding exons is located in the second extracellular loop, and the third coding exon is responsible for the remainder of the protein, including the last three transmembrane domains and the intracellular carboxyl tail. The binding pocket has been proposed to comprise several of the transmembrane domains *(Topham et al. 1998; Mouledous et al. 2000)*. The initial gene structure identified five exons, with a noncoding exon preceding and following the three coding exons; other work has identified two mini-exons between the first and second coding exons that are alternatively spliced for a total of seven *(Mogil J.S., Pasternak G.W. 2001)*.

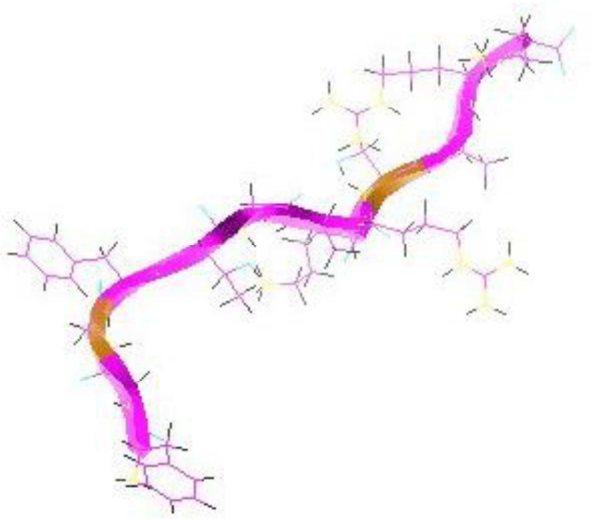
The regional distribution of NOP receptor have been well described *(Bunzow J.R. et al. 1994; Fukuda K. et al. 1994; Mollereau et al., 1994; Wick et al. 1994; Lachowicz J.E. et al. 1995)*. Regions with NOP1 receptor binding typically express NOP1 mRNA as well, although the levels of mRNA and binding do not always match very closely. Regions with high levels of NOP1 binding/mRNA include the cortex, anterior olfactory nucleus, lateral septum, hypothalamus, hippocampus, amygdala, central gray, pontine nuclei, interpeduncular nucleus, substantia nigra, raphe complex, locus coeruleus, and spinal cord *(Mogil J.S., Pasternak G.W. 2001)*.

1.3.3.2 Nociceptin

N/OFQ is a heptadecapeptide with some interesting structural homologies to the classical opioid peptide dynorphin A (Fig 14). Both peptides are comprised of 17 amino acids bounded by pairs of basic amino acids important in their production from their precursors. Furthermore, both have internal pairs of basic amino acids, raising the possibility of further processing. The opioid peptides share a YGGF motif, where the fifth amino acid is either leucine or methionine. The amino terminus of N/OFQ is a phenylalanine instead of a tyrosine, followed by GGF. Finally, both peptides contain the same last two amino acids at the carboxyl terminus. Despite

these similarities, the peptides are functionally quite distinct. N/OFQ has no appreciable affinity for any of the opioid receptors *(Mogil J.S., Pasternak G.W., 2001).*

a)



b)

Structures of Some Opioid Peptides; Comparison with Nociceptin

Nociceptin	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser Ala-Arg-Lys-Leu-Ala-Asn-Gln
Dynorphin A 1-17	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Arg-Asn-Gln
γ-Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser Gln-Thr-Pro-Leu-Val-Thr-Leu
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Endomorphin 1	Tyr-Pro-Trp-Phe
Endomorphin 2	Tyr-Pro-Phe-Phe

Figure 14 **a)** Three-dimensional representation of the nociceptin.
b) Structure of Nociceptine compared with some Opioid Peptides

Like all bioactive peptides, N/OFQ is generated from a larger precursor peptide, prepro N/OFQ (ppN/OFQ) that has been cloned from mouse, rat, and human (Fig. 15) *(Meunier et al. 1995; Pan Y-X et al. 1996a; Reinscheid R.K. et al. 2000).* Overall, there is high interspecies homology, with 80% identity among the three organisms.

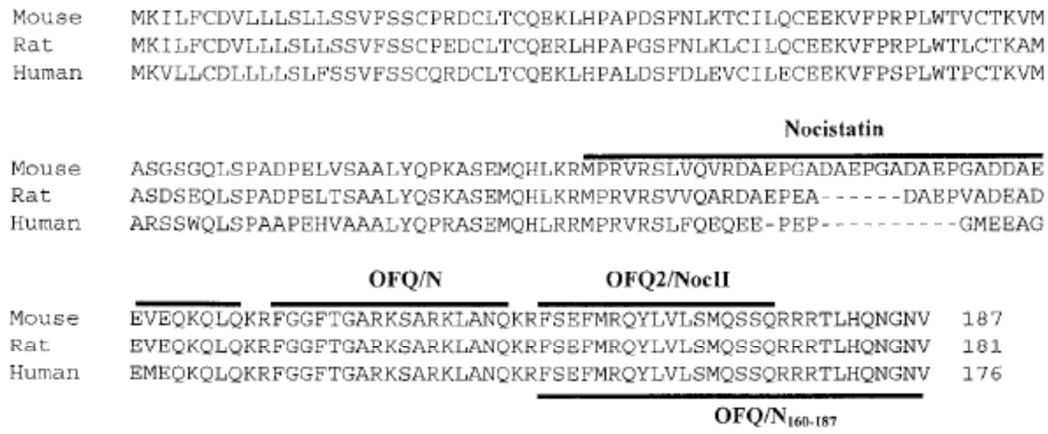


Figure 15 Schematic of the prepro-OFQ/N gene *(Mogil J.S., Pasternak G.W., 2001).*

The primary structure of the precursor protein contains the typical structural elements of a neuropeptide precursor. It starts with an amino terminal signal peptide necessary for its secretion. The N/OFQ sequence is flanked by pairs of Lys-Arg residues, indicating that its maturation requires trypsin-like cleavages. The C-terminus of the precursor protein downstream of the N/OFQ sequence is conserved among the different species genes and could generate either a 28- residue long peptide or, after cleavage at the Arg triplet, a 17-residue long peptide, whose terminal amino acids are the same as these of N/OFQ. These two peptides were synthesized but were unable to either bind or activate the NOP receptor *(Civelli O. 2008).* It has been reported that the 17-amino acid peptide (termed NocII or OFQ II)

exhibits some effect on locomotion and pain perception *(Florin S. et al. 1997; Rossi G.C. et al. 1998).*

The bovine precursor harbors an additional pair of basic amino acids N-terminal to the N/OFQ sequence that could give rise to a 19-amino acid peptide *(Okuda-Ashitaka E. et al. 1998).* This putative was isolated from bovine bran and has been reported to possess an anti-N/OFQ activity because it was able to block N/OFQ induced allodynia and hyperalgesia *(Okuda-Ashitaka E. et al. 1998).* This peptide was named nocistatin and acts via a receptor different from the NOP receptor. The active part of nocistatin was found to reside in its C-terminal hexapeptide that is also the only conserved structure between all mammalian N/OFQ precursors. The bovine form of nocistatin appears to be species-specific because the human, rat, mouse, and porcine precursor lack the pair of basic amino acids that is used for processing in the bovine precursor protein. The fact that OFQ II or nocistatin require their own receptors but that none have been identified that would exhibit selectivity for these two peptides indicate that these peptides may not be neuropeptides that act through a traditional GPCR system *(Civelli O. 2008).*

The localization of N/OFQ corresponds reasonably well with the NOP1 receptor. As with the receptor, N/OFQ immunoreactivity and mRNA levels detected using in situ hybridization are closely correlated. N/OFQ is found in lateral septum, hypothalamus, ventral forebrain, claustrum, mammillary bodies, amygdala, hippocampus, thalamus, medial habenula, ventral tegmentum, substantia nigra, central gray, interpeduncular nucleus, locus coeruleus, raphe complex, solitary nucleus, nucleus ambiguous, caudal spinal trigeminal nucleus, and reticular formation, as well the ventral and dorsal horns of the spinal cord *(Neal C.R. et al. 1999).*

1.3.3.3 Pharmacological Effects of Nociceptin/Orphanin FQ

The N/OFQ receptor was shown to induce a variety of intracellular effects. First N/OFQ receptor was shown to induce an inhibition of adenylyl cyclase in CHO cells transfected with the NOP receptor *(Meunier et al. 1995; Reinscheid et al. 1995).* Next, N/OFQ was found to modulate cellular excitability increasing inwardly rectifying K-conductance in dorsal raphe nucleus neurons *(Vaughan C.W., Christie M.J. 1996)* and in the arcuate nucleus *(Wagner E.J. et al. 1998)*; to increase K-conductance in periaqueductal gray neurons *(Vaughan et al. 1996)* and in locus coeruleus neurons; to couple to G protein-activated K channels; to inhibit voltage-gated calcium channels in freshly dissociated CA3 hippocampal neu- rons; to inhibit T-type Ca channels in sensory neurons; and to inhibit N-type Ca channels in SH-SY5Y cells. Also, the N/OFQ receptor appears to couple to K channels in Xenopus oocytes. Furthermore, N/OFQ has been shown to inhibit the release of glutamate and GABA from nerve terminals, to block acetylcholine release from retina and parasympathetic nerve terminals, to inhibit synaptic transmission and long-term potentiation in the hippocampus, to suppress dopamine release in the nucleus accumbens, and to inhibit tachykinin and calcitonin gene-related peptide release from sensory nerves. N/OFQ was demonstrated to activate mitogen-activated protein kinase in re- ceptor transfected CHO cells. Together, these results show that N/OFQ is able to modulate the biochemical properties of cells, alter the electrophysiological properties of neurons and to affect their transmitter reease. Importantly, none of the effects of N/OFQ described were inhibited by opiate antagonists, emphasizing the pharmacological difference between the opioid and the N/OFQ systems *(Civelli O. 2008).*

1.3.3.4 Nociceptin and pain

There is a wealth of evidence showing that N/OFQ has pronociceptive and antinociceptive effects depending on the drug dose, test, and route/side of injection used. N/OFQ at low doses in the pmol and fmol ranges displays pronociceptive action, whereas N/OFQ exhibits antinociceptive potential when administered at higher doses in the nmol range, *(Mogil J.S., Pasternak G.W. 2001; Yamamoto T. et al. 1999;* Tab, 3 Prezwloka). Both effects induced by N/OFQ have been suggested to be mediated by the NOP receptor.

Rout of injection	Nociceptin		Dynorphin	
	pmol/fmol	nmol	pmol/fmol/nmol	<nmol
Subcutaneous	• ND	• No changes in pain sensitivity (Obara et al. 2005)	• Analgesia (Ko et al., 2000)	• No changes in pain sensitivity (Ko et al. 2000)
Intraplantar	• Hyperalgesia (Inoue et al., 1998) • Itch (Andoh et al. 2004)	• No changes in pain sensitivity (Obara et al., 2005)	• Analgesia (Beyer et al., 1997)	• ND
Intrathecal	• Hyperalgesia • Allodynia • Decreased locomotor activity (Hara et al., 1997; Reinscheid et al., 1995; Zhang et al., 1997; Zhu et al., 1998; Bertorelli et al., 1999)	• No changes in pain sensitivity (Courteix et al., 2004; Vanderah et al., 1998; Obara et al., 2005) • Analgesia (Jhamandas et al., 1998; Tian et al., 1997a; Wang et al., 1999a; Xu et al., 1996, 2000)	• Analgesia (Jhamandas et al., 1986; Przewlocki et al., 1983a,b)	• Hyperalgesia • Allodynia • Nociceptive behavior • Motor dysfunction (Tan-No et al. 2002; Laughlin et al. 1997; Vanderah et al. 1996; Stevens and Yaksh 1986)
Intracerebroventricular	• Hyperalgesia • Decreased locomotor activity (Meunier, 1997; Suaudeau et al., 1998; Reinscheid et al., 1995)	• No changes in pain sensitivity (Zhu et al., 1998; Vanderah et al., 1998; Darland et al., 1998; Grisel et al., 1996) • Hyperalgesia (Citterio et al., 2000)	• Analgesia (Kuzmin et al., 2006)	• Motor dysfunction • (Nakazawa et al. 1985)
Into periaqueductal grey	• Hyperalgesia (Wang et al., 1998)	• ND	• No changes in pain sensitivity (Han and Xie, 1984)	• No changes in pain sensitivity (Han and Xie, 1984)
Receptor involvement	• NOP receptor (Inoue et al., 1998, 1999)	• NOP receptor (Inoue et al., 1998, 1999)	• KOP receptor (Chavkin et al., 1982)	• NMDA receptor (Laughlin et al., 1997; Vanderah et al., 1996) • Bradykinin receptor (Lai et al., 2008)

Table 3 A comparison of the effects induced by application of nociceptin and dynorphin on acute pain sensitivity and motor function/activity *(Mika J. et al. 2011).*

In behavioral studies, intracerebroventricular (i.c.v.) administration of N/OFQ was found to display hyperalgesic effects that are observed as reduced latencies in the hot plate test *(Meunier et al. 1995; Suaudeau C. et al. 1998)* and the tail-flick test *(Suaudeau C. et al., 1998; Reinscheid R.K. et al. 1995)* and as decreased locomotor activity in mice *(Reinscheid et al., 1995).* Also, N/OFQ that was microinjected into the periaqueductal gray increased pain sensitivity in rats *(Wang H. et al., 1998).*

These effects were likely mediated by the NOP receptor based on the results of a study that has shown that the pronociceptive action of N/OFQ is not present in NOP knockout mice and that antisense oligonucleotides targeting the NOP receptors prevent the N/OFQ effect *(Calo G. et al. 2000)*. On the other hand, it was demonstrated that N/OFQ administered i.c.v. in the nmol dose range was ineffective in significantly altering the response to noxious stimuli *(Zhu C.B. et al. 1998; Vanderah T.W. et al. 1998)*. Although a consensus on the precise function of N/OFQ in the modulation of nociception has not been reached, a large body of evidence indicates that supraspinal injection of N/OFQ does not affect pain sensitivity *(Grisel J.E. et al. 1996)*. Interestingly, it has also been suggested that the pronociceptive effects produced by i.c.v. administration of N/OFQ may provide an explanation for the anti-opioid action of N/OFQ *(Mogil et al., 1996)*. This phenomenon was confirmed by experiments demonstrating that N/OFQ was able to alter the analgesic effectiveness of morphine and other opioids.

In contrast to the pronociceptive effects induced by i.c.v. injection of N/OFQ, at the spinal level, i.t. administered N/OFQ generally has been found to produce antinociceptive responses that are similar to classical opioid receptor agonists without inducing signs of sedation or motor impairment (tab. 3). However, i.t. N/OFQ at doses lower than those exhibiting analgesic potential induced hyperalgesia, allodynia and impaired locomotor activity. Thus, N/OFQ at low doses in the pmol and fmol ranges displayed pronociceptive action resulting from the activation of NOP receptors at the spinal level and exhibited antinociceptive potential at higher, nmol doses. However, it should also be noted that some authors reported that N/OFQ applied i.t. at nmol doses had no effect by itself. Interspecies and even interstrain differences, disparate doses and different testing procedures may ac- count for the conflicting results reported with N/OFQ and other NOP receptor ligands in the spinal cord *(Mika J. et al. 2011)*.

It has been clearly documented that the induction of chronic pain states, especially neuropathy, is associated with an upregulation of N/OFQ synthesis, as indicated by enhanced expression of pN/OFQ protein and/or mRNA (Dubner and Ruda, 1992; Kajander et al., 1990; Malan et al., 2000; Mika et al., 2010; Fig. 16).

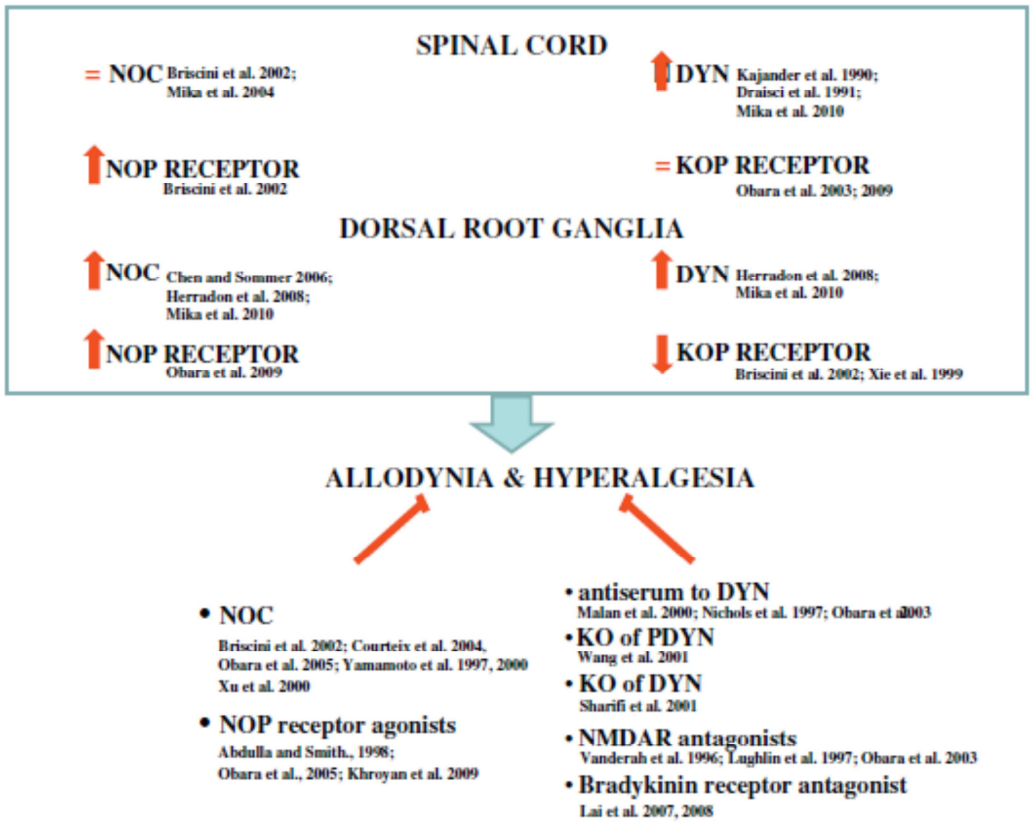


Figure 16 A comparison of the changes in the levels of mRNA and/or protein for nociceptin (N/OFQ), dynorphin (DYN), nociceptin-opioid peptide (NOP) receptor and kappa-opioid peptide (KOP) receptor in the ipsilateral part of the dorsal spinal cord and ipsilateral dorsal root ganglia under neuropathic pain conditions *(Mika J. et al. 2011).*

Many studies have supported the suggestion that the analgesic effectiveness of the N/OFQ and NOP receptor ligands is increased in neuropathic conditions and that this effect is associated with upregulation of NOP receptor mRNA in the spinal cord and DRG after nerve injury. Moreover, at the spinal level, it is well-documented that N/OFQ produces antinociception through presynaptic (DRG) and postsynaptic (spinal cord) mechanisms. The increase in N/OFQ in DRG neurons after axotomy as well as after sciatic nerve ligation is potentially related to the enhanced spinal N/OFQ release from afferent neurons and, therefore, may be an additional factor in this regulatory circuit. This effect is in contrast to the classical opioid receptor agonists, such as morphine, which are less effective in neuropathy than in acute pain conditions. Behavioral experiments have demonstrated that N/OFQ administered i.t. alleviates allodynia and hyperalgesia resulting from injury of the sciatic nerve *(Mika J. et al. 2011).*

1.3.3.5 Anxiety, stress and depression

The observation that N/OFQ is able to reverse stress-induced analgesia *(Mogil J.S. et al. 1996)* and that the NOP receptor is expressed in brain centers known to modulate anxiety prompted investigations into the effects of N/OFQ on stress as it relates to anxiety. Central administration of N/OFQ was found to exert profound anxiolytic effect comparable to classic anxiolytic drugs such as diazepam *(Jenck F. et al. 1997).* Low nanomolar doses of N/OFQ decreased behavioral measures of anxiety in rats using the light–dark box and elevated plus maze paradigms. Similar to the biphasic effect of diazepam, higher doses of N/OFQ also showed motor impairment. These findings were later confirmed with mice in the elevated plus maze paradigm *(Gavioli E.C. et al. 2002)* or the hole-board exploration test *(Kamei J. et al. 2004)* and in the defense test battery *(Griebel G. et al. 1999)*, although in the latter paradigm, the effects were observed only after very high stress. Altogether, these experiments provided evidence that the N/OFQ system might be involved in modulation of stress as it relates to anxiety. The mechanism by which the N/OFQ system modulates stress is not fully understood but implicates regulation of

the hypothalamic–pituitary–adrenal (HPA) axis. N/OFQ KO mice exhibit elevated plasma corticosterone levels under both resting and post-stress conditions *(Reinscheid R.K., Civelli O. 2002),* and N/OFQ administrations reverse transient increases in plasma corticosterone precipitated by stressors such as intracerebroventricular injections.

The first indication that the N/OFQ system may be involved in modulating depressive states of behavior came from the observation that rat pups who were separated from their mothers exhibit elevated levels of N/OFQ expression in adolescence. That the N/OFQ system may have a role in depression-like symptoms is further indicated by the finding that N/OFQ levels are elevated in postpartum depressive women but, more importantly, by studies that showed that N/OFQ antagonists could produce antidepressant effects, such as reduced immobility time in the mouse forced-swim test, which reflects behavioral despair. Moreover, NOP KO mice also display reduced immobility time in the same assay. The mechanism for the antidepressive effects of N/OFQ antagonists is unknown. N/OFQ is able to attenuate serotonin release from dorsal raphe neurons and rat cortical serotonergic terminals, thus acting to reduce serotonin levels. Conversely, N/OFQ antagonists are able to block the N/OFQ-induced inhibition of noradrenaline and serotonin release from cortical synaptosomes *(Civelli O. 2008).*

1.3.4 Dynorphin system

Dynorphin system is part of Endogenous Opioid System including the dynorphin (DYN) peptide and his receptor KOP. This system is widely involved in neuropathic pain, but its role is complex and not completely clear yet.

1.3.4.1 Dynorphin

In 1979, *Goldstein A. et al. 1979* described the opioid properties of a tridecapeptide, which they had first isolated from porcine pituitary four years earlier *(Cox B.M. et al. 1975; Teschemacher H. et al. 1975)*. The first five amino acids of this peptide (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile- Arg-Pro-Lys-Leu-Lys) represent Leu-enkephalin. To denote its extraordinary potency, the natural peptide was named "dynorphin" from the Greek dynamis=power and the ending orphin indicating its opioid nature. Two years later, the complete sequence of 17 amino acids was identified *(Goldstein A. et al. 1981)* (Fig. 17) This peptide was renamed to dynorphin A after the isolation of the larger form dynorphin-32 which consists of the original 17 amino acids at its amino-terminus and a novel Leu-enkephalin containing tridecapeptide now termed dynorphin B (=rimorphin) at the carboxy-terminus. The two peptides are linked by a pair of basic amino acids (Lys-Arg), which indicate a potential processing site *(Fischli W. et al. 1982a,b)*. A smaller bioactive form of dynoprhin A, dynorphin 1-8, was described in 1980 *(Minamino N. et al. 1980)*. The first five amino acids (i.e. those representing Leu-enkephalin) were proposed as essential for binding to opioid receptors *(Chavkin C., Goldstein A. 1981)*. Characterization of the precursor of dynorphins (Dyn), prodynorphin (pDyn, also termed proenkephalin B) at the mRNA *(Kakidani H. et al.1982)* and protein level *(Watson S.J. et al. 1983)* also revealed the presence of α- and β-neoendorphin *(Minamino N. et al. 1981)*, leumorphin (=dynorphin B 1-29; assembled from dynorphin B and the C-terminal C-peptide) as well as a number of biologically inactive fragments, which do not contain the Leu-enkephalin motif.

DYN have increasingly been thought to play a regulatory role in numerous functional pathways of the brain. In line with their localization in the hippocampus, amygdala, hypothalamus, striatum and spinal cord, these functions are related to learning and memory, emotional control, stress response and pain. Pathophysiological mechanisms that may involve DYN/kappa opioid receptors (DYN/KOP) include epilepsy, addiction, depression, schizophrenia, and chronic pain *(Schwarzer C. 2009)*.

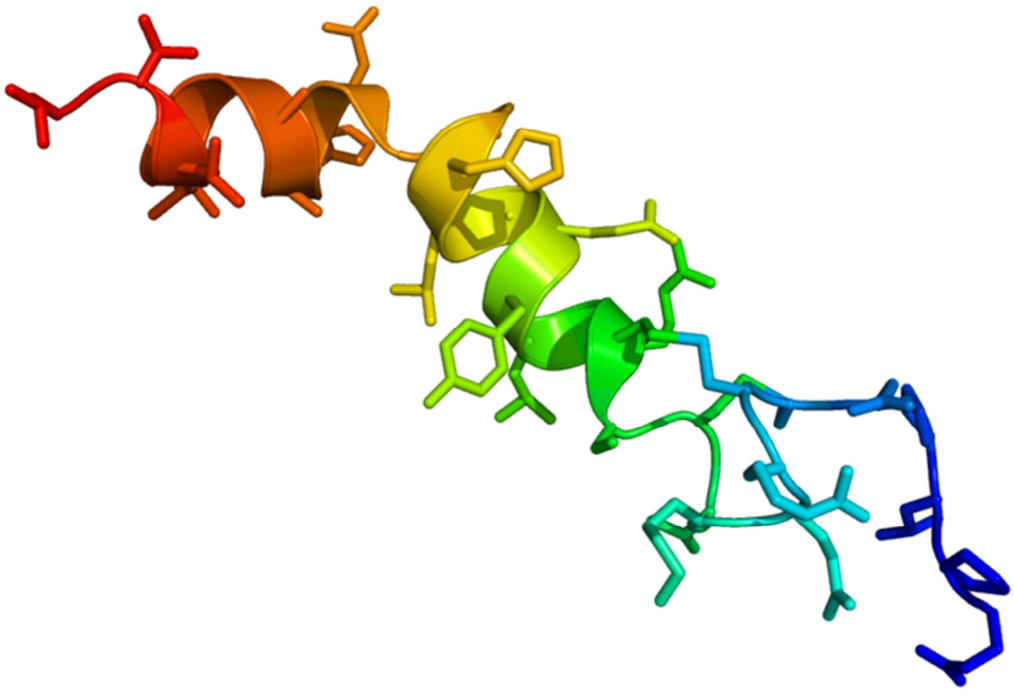


Figure 17 3D structure of DYN A (1-17)

1.3.4.2 Dynorphin gene and mRNA

The pDYN gene contains four exons (1–4) and three introns (A,B,C) in humans and rodents *(Horikawa S. et al. 1983; Douglass J. et al. 1989; Sharifi N. et al. 1999)*. While exons 1 and 2 encode for the majority of the 5′-untranslated region, exons 3 and 4 contain the entire coding sequence (Fig. 13) *(Schwarzer C. 2009)*. Several promoter elements have been identified within the rat pDYN promoter. The four CRE sites observed in the rat promoter were thought to be the most important, perhaps being responsible for the excitation-dependent regulation of pDYN expression *(Douglass J. et al. 1994)*. In terms of suppression of pDYN expression, the downstream regulatory element (DRE) and its Ca2+-regulated transcriptional repressor DREAM was suggested to be important *(Carrion A.M. et al. 1999; Campos D. et al. 2003)*. DREAM appears to play a crucial role in the regulation of pDYN expression in neuropathic and inflammatory pain. Low concentrations of DYN acting on KOP located on spinal projection neurons produce analgesic effects. In contrast, a single intrathecal injection of a higher dose of DYN produces long-lasting allodynia in mice and rats. This puzzle was solved by identification of NMDA receptors as target of high concentrations of DYN *(Vanderah T.W. et al. 1996; Laughlin T.M. et al. 1997)*. Noteworthy, knockout of DREAM, leading to increased expression of pDyn markedly reduces a broad spectrum of acute and chronic pain related behaviours *(Cheng H.Y. et al. 2002)*.

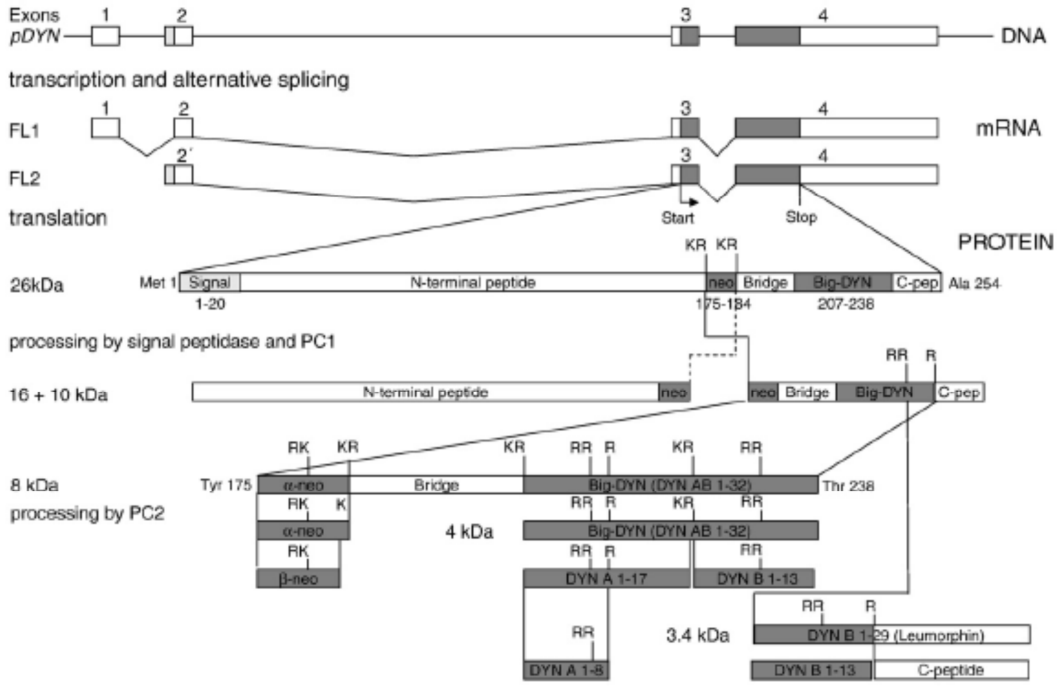
Seven pDYN mRNA splice variants have been isolated from human brain *(Horikawa S. et al. 1983; Telkov M. et al. 1998; Nikoshkov A. et al. 2005)*. Two of the transcripts, termed FL1 and FL2, contain the entire coding sequence of pDYN (Fig. 18). The predominant form FL1 is highly expressed in limbic structures such as the nucleus accumbens and amygdala, while the expression of FL2 is restricted to a few brain areas including the claustrum and hypothalamus *(Nikoshkov et al., 2005)*. These two transcripts differ in their 5′-non-coding region. FL1 transcripts are initiated somewhat upstream of the proposed transcription initiation site *(Douglass J. et al. 1994)*. FL2 contains a novel second exon, which extends the originally described exon 2 and is initiated within intron A close to a site previously detected in

embryonic brain *(Telkov M. et al. 1998)*. The exons comprising FL1 and FL2 are highly conserved in human, mouse and rat genomes *(Schwarzer C. 2009)*.

1.3.4.3 Processing of Dynorphin

Like all other neuropeptides, DYN are processed from a large biologically inactive precursor protein. The first evidence for differential processing of pDYN was observed in the lobes of the pituitary. Processing of pDYN requires the endopeptidases, termed prohormone convertases (PC), PC1 and PC2 and carboxypeptidase E (Fig.18). PC1 was proposed to cut at the carboxy side of three of the seven pairs of basic amino acids. The primary target is the Lys-Arg pair N-terminal to α- neoendorphin, yielding a 10 kDa C-terminal fragment containing all known pDYN-derived peptides (Fig. 18). A minor alternative pathway may be the proteolytic cleavage at the Lys-Arg pair C-terminal to α-neoendorphin. In a second step, a carboxyterminal fragment of about 2 kDa is cleaved at a single Arg, yielding an 8 kDa intermediate product *(Dupuy A. et al. 1994)*. Further processing requires PC2, producing biologically active peptides including α-neoendorphin, big- DYN, leumorphin, DYN A 1-17 and 1-8 and DYN B (Fig.13). This processing is enhanced by the presence of carboxypeptidase E *(Day R. et al. 1998)*.

In vitro studies on the ability of different pDyn-derived peptides to activate kappa opioid receptors suggested a rank order of potency with Dyn A1-17 > (10–20 times) big- DYN=DYN B=DYN B 1-29=α-neo-endorphin>(10–20 times) Dyn A 1-8 = β neo-endorphin *(James I.F. et al. 1984)*. Differential processing of pDyn was also observed in the brain. Electron microscopy showed the coexistence of pDyn and Dyn within the same axon and even individual vesicles *(Yakovleva T. et al. 2006)*. While the classical model suggests initiation of processing of pDYN in the trans-Golgi network, a newly hypothesized model suggests transport of pDYN to the synapse and initiation of processing in response to external stimuli. Such a regional regulation of trafficking and processing at synapses may provide local regulation of synaptic transmission *(Yakovleva T. et al. 2006) (Schwarzer C. 2009)*.



-Dynorphin-like peptides derived from the prodynorphin precursor.

Peptide	Amino acid sequence
[a]Dynorphin $A_{(1-8)}$	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile^{8}
[b]Dynorphin $A_{(1-13)}$	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys^{13}
[c]Dynorphin $A_{(1-17)}$	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln^{17}
[d]Dynorphin $A_{(1-32)}$	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr^{32}
[d]Dynorphin $B_{(1-13)}$	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr^{13}
[e]Leumorphin	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val^{29}
[f]α-neoendorphin	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys^{10}
[g]β-neoendorphin	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro^{9}

Citations: a, (Minamino et al., 1980); b, (Goldstein et al., 1979); c, (Goldstein et al., 1981); d, (Fischli et al., 1982); e, (Kakidani et al., 1982); f, (Kangawa et al., 1981); g, (Minamino et al., 1981).

Figure 18 Biosynthesis of pDYN derived peptides. The entire coding sequence of pDYN is contained in exons 3 and 4 (dark grey shading) of the pDYN gene.

Several differentially spliced transcripts are derived from this single gene, however only the two full-length mRNAs FL1 and FL2 are found in humans and rodents. These two splice variants differ only in the 5´- non-coding region with FL2 being transcribed from an extended exon 2 (light grey). An identical 254-amino acid preprodynorphin is translated from both mRNAs. The first 20 amino acids represent the signal peptide, responsible for targeting the protein towards the endoplasmatic reticulum. This peptide is immediately cleaved by the signal peptidase, resulting in pDYN. Further processing is differentially regulated in distinct brain regions, resulting in pDYN as well as mature peptides in axon terminals. Maturation is dependent on prohormone convertases PC1 and PC2. Processing of the mature peptides at the paired arginine residues yields Leu enkephalin from β-neoendorphin, DYN A and DYN B *(Schwarzer C. 2009)*.

1.3.4.4 KOP receptor

The kappa-opioid receptor is a G protein-coupled receptor (Fig. 19) and stimulation of the kappa-opioid receptor leads to the inhibition of adenylyl cyclase *(Attali B. et al. 1989; Konkoy C.S., Childers S.R. 1989; Prather P.L. et al. 1995).* The kappa-opioid receptor also plays a role in the regulation of calcium currents. The kappa-opioid receptor is coupled to an N-type calcium channel and stimulation of the kappa-opioid receptor inhibits calcium currents *(Tallent M. et al. 1994).* N-type calcium channels are expressed on presynaptic terminals and play a critical role in the release of neurotransmitters *(Evans R.M., Zamponi G.W. 2006).*
Therefore, dynorphin-like peptides may inhibit the release of neurotransmitters by inhibiting N-type calcium channels. Furthermore, kappa-opioid receptor agonists have been shown to stimulate inwardly rectifying (i.e., ion conductance depends on membrane potential) potassium channels *(Henry D.J. et al. 1995; Lewin B. et al. 2007).* The inward rectifying potassium current is a flow of potassium ions from the outside to the inside of the cell *(Lu Z. 2004).* The potassium conductance increases when the membrane potential becomes mor enegative and the potassium conductance decreases when the membrane depolarizes. The inward rectifying potassium channels play an important role in maintaining the resting potential of neurons *(Lu Z. 2004).*

Pharmacological studies have provided some evidence for the existence of multiple kappa-opioid receptor subtypes, however, so far only one kappa-opioid receptor has been cloned *(Bruijnzeel A.W. 2009).* The kappa-opioid receptor has been cloned from many species including rat, mouse, guinea pig, and human *(Meng F. et al. 1993; Simonin F.et al. 1995; Xie G.X. et al. 1994; Yasuda K. et al. 1993; Zhu J. et al. 1995). Attali B. et al. (1982)* described two types of kappa-opioid receptors in the spinal cord of guinea-pigs. Evidence has been provided for U-69593 sensitive high-affinity binding sites (κ1) and U-69593 insensitive low affinity binding sites (κ2) *(Zukin R.S. et al., 1988).* High levels of kappa-opioid receptormRNA have been detected in the ventral tegmental area, substantia nigra, nucleus accumbens, caudate putamen, claustrum, endoperiform nucleus, various hypothalamic nuclei, and the amygdala of the rat brain *(Meng F. et al. 1993).* A

similar expression profile has been detected in the human brain *(Simonin F. et al. 1995; Zhu J. et al. 1995).*

Figure 19 3D structure of the KOP receptor complexed with the JDTic selective ligand.

1.3.4.5 Dynorphin and neuropathic pain

Unlike the other two classes of known endogenous opioid peptides, the enkephalins and endorphins, intrathecal administration of dynorphin — whose N-terminal sequence is identical to [Leu]enkephalin — does not elicit robust antinociception. Rather, dynorphin and those derivatives that lack the crucial N-terminal tyrosine residue necessary for interaction with opioid receptors produce motor impairment and paralysis (*Lay J. et al. 2001*). Intrathecally administered dynorphin and its non-opioid derivatives produce hindlimb paralysis *(Stevens C.W. et al. 1986; Faden A.I., Jacobs T.P. 1984)* and enhanced sensitivity to sensory stimuli; at paralytic doses, these peptides are neurotoxic, depleting sensory neurons, motor neurons, and interneurons in the spinal cord (*Long J.B. et al. 1988*), and potentiating excitatory neurotransmitter release (*Faden A.I. 1992; Skilling S.R. et al. 1992*). At non-paralytic doses, dynorphin elicits long-lasting hyperresponsiveness to innocuous mechanical and noxious thermal stimuli (*Vanderah T.W. et al. 1996; Laughlin T.M. et al. 1997*). The essentially equivalent actions of dynorphin and its non-opioid derivatives strongly suggest that the effects of dynorphin are non-opioid. Increased responsiveness to innocuous and noxious sensory stimuli are reminiscent of signs of experimental and clinical neuropathic pain, suggesting that endogenous dynorphin is a key mediator of some aspects of neuropathic pain states (*Lay J. et al. 2001*).

Peripheral nerve injuries are accompanied by an increase in spinal dynorphin (*Draisci G. et al. 1991*) especially in spinal interneurons (*Botticelli L.J. et al. 1981*). Within five days subsequent to nerve injury, dynorphin concentrations are elevated in the dorsal horn (i.e., where afferent axons terminate), peaking at day ten and persisting past day twenty post injury (*Kajander K.C. et al. 1990*); levels of mRNA for preprodynorphin correspondingly rise (*Draisci G. et al. 1991*). Complete nerve transection or nerve crush injury, however, produces only minimal changes in preprodynorphin mRNA levels (*Draisci G. et al. 1991*), suggesting that sustained afferent discharge is important for initiating the upregulation of spinal dynorphin. A study have indicated significant increases in spinal dynorphin-like content in the ipsilateral dorsal quadrant of the spinal cord after injury to the L5 and L6 spinal nerves *(Malan T.P. et al. 2000).* A bilateral increase in immunologically detected

dynorphin content is also observed in a sciatic cryoneurolysis model of neuropathic pain *(Wagner R. et al. 1993)*. These studies suggest that the enhanced synthesis and release of spinal dynorphin after nerve injury may be common in chronic pain.

In accordance with its classification as an endogenous opioid it has generally been concluded that elevated concentrations of spinal dynorphin function to dampen chronic nociceptive input after nerve injury (*Draisci G. et al. 1991; Kajander K.C. et al. 1990*). Nevertheless, considerable evidence reveals that dynorphin is actually pronociceptive in chronic pain states. For instance, the spinal administration of anti-dynorphin antiserum reduces neurological impairment after nerve injury (*Cox B.M. et al. 1985*). Even the intrathecal injection of anti-dynorphin antiserum blocks the increased sensitivity to noxious thermal and innocuous mechanical stimuli that commonly accompanies injury to spinal nerves, but that the antiserum does not alter normal sensory thresholds in non-injured animals (*Nichols M.L. et al. 1997*). These findings support the hypothesis that the upregulation of spinal dynorphin is pronociceptive and important in the maintenance of the experimental neuropathic pain state (*Lay J. et al. 2001*). Further confirmation comes from experiments using prodynorphin knockout mice . These mice generally show normal responses to innocuous and noxious stimuli, thereby suggesting that basal levels of spinal dynorphin do not substantially participate in sensory thresholds. They also develop, as do their wild-type litter mates, neuropathic pain within two to three days after nerve injury, manifesting the typical increase in sensitivity to innocuous mechanical and noxious thermal stimuli. Unlike their wild-type litter mates, however, the prodynorphin knockout mice fail to sustain the neuropathic pain state for extended periods. By ten days after injury, the knockout mice are no more sensitive than they were prior to injury. In contrast, wild-type animals display neuropathic pain past day fourteen (*Wang Z. et al. 2001*). These data suggest that spinal dynorphin does not contribute to initial pain onset, but rather is critical for the maintenance of neuropathic pain (*Lay J. et al. 2001*). Correspondingly, early after nerve injury (ca two days), anti-dynorphin antiserum is ineffective blocking neuropathic pain while it becames effective ten days post injury, when spinal dynorphin levels are at their peak (*Wang Z. et al. 2001*).

1.3.4.6 Dynorphin and non-opioid systems

Several investigators have demonstrated that dynorphin-induced neurological damage, such as hindlimb paralysis, loss of tail-flick reflex, and loss of neuronal cell bodies, can be prevented by antagonists and modulators of the NMDA receptor, which is known to be an important mediator of neuropathic pain states *(Bakshi R., Faden A.I. 1990; Woolf C.J., Thompson S.W.N. 1991)*. Similarly, the long-lasting hyperresponsiveness to both innocuous and noxious stimuli that is elicited by intrathecal dynorphin is not blocked by the opioid receptor antagonist naloxone, but is prevented by pretreatment with the NMDA receptor antagonist *(Vanderah T.W. et al.1996; Laughlin T.M. et al. 1997).*

The interaction of endogenous dynorphin and NMDA receptors in promoting neuropathic pain may be either direct or indirect. Studies conducted through displacement of highly-selective NMDA-channel radioligand, showed that Dynorphin is a positive allosteric modulator of the binding of the competitive NMDA receptor antagonist to the glutamate binding site *(Dumont M., Lemaire S. 1994)*. Thus, both direct and indirect binding analyses indicate that dynorphin A associates more strongly with NMDA receptors that are in the closed channel state. shortening the mean open time and decreasing the probability of channel opening, but does not seem to affect single channel conductance. Thus, although we cannot entirely rule out a direct, excitatory interaction between dynorphin and NMDA receptors, most evidence argues against direct interaction as a predominant mechanism for the excitatory actions of dynorphin, implying that non-opioid actions of dynorphin arise from interactions not involving NMDA receptors *(Lay J. et al. 2001).*

The hypothesis that the biological roles of dynorphin extend beyond opioid and NMDA receptors is supported by the finding that the application of dynorphin to the rat hippocampus results in a localized, dose dependent release of glutamate and aspartate *(Faden A.I. 1992)*. Thus, dynorphin may indirectly potentiate NMDA receptor activity by enhancing the release of excitatory neurotransmitters, thereby resulting, for example, in hindlimb paralysis and neuronal cell loss *(Lay J. et al. 2001).* It is feasible that dynorphin presynaptically promotes the release of these

neuropeptides by facilitating the rise in intracellular calcium concentration that would accompany activation of NMDA receptors. Indeed, neurotransmitter release depends on the transient increase in calcium that facilitates the interaction of proteins that mediate the docking and fusion of synaptic vesicles to the plasma membrane at the axon terminal *(Catterall W.A. 1998)*. The novel mechanism of dynorphin-induced increase in [Ca2+]i is currently under investigation. Many potential calcium dependent mechanisms could facilitate neurotransmitter release by dynorphin (Fig. 20). For example, NMDA receptor mediated currents are potentiated by protein kinase C and its activators *(Chen L., Huang L.Y. 1992)*. In addition, many voltage-gated calcium and potassium conductances are modulated by calcium through calmodulin, which also regulates the activity of a number of kinases that have been implicated in the regulation of neurotransmitter release *(Llinas R. et al. 1991; Nichols R.A. et al. 1990)* and in the phosphorylation of synaptic proteins *(Nayak A.S. et al. 1996)*. A recent study of cultured embryonic spinal cord neurons shows that dynorphin A(1-13) stimulates an acute, transient increase in [Ca2+]i *(Hauser K.F. et al. 1999)*. The excitatory effects of dynorphin are therefore apparent in both presynaptic (dorsal root ganglion) and postsynaptic (spinal cord) neurons. Prolonged exposure of these cells to dynorphin not only leads to an elevated basal level of [Ca2+]i, but also a significant loss of neurons; both of these effects are prevented by NMDA antagonists, suggesting that the neurotoxic effects of dynorphin are calcium dependent and mediated by NMDA receptors. These data support the notion that continuous exposure to dynorphin potentiates NMDA receptor activity, in accord with the neurotoxicity of intrathecal dynorphin to spinal cord neurons as mentioned above (*Long J.B. et al. 1988)*.

Concluding, under normal physiological conditions, spinal dynorphin plays a minimal role in the regulation of sensory thresholds, a conclusion that is supported by prodynorphin knockout mice *(Wang Z. et al. 2001)*. Rather, the opioid action of dynorphin is to suppress noxious inputs. However, under pathological conditions resulting from injury to peripheral nerves, the upregulation of spinal dynorphin accompanies the development of chronic pain states that can be blocked by anti-dynorphin antiserum, suggesting that elevated spinal dynorphin is required for maintaining neuropathic pain (Fig. 20). The mechanisms that mediate the

upregulation of spinal dynorphin are unknown. Evidence from many laboratories consistently shows that neuronal excitation by dynorphin is mediated by an increase in intracellular calcium, which may act to enhance the release of transmitters from sensory primary afferent neurons or to potentiate NMDA receptor activity in spinal cord cells. The former possibility might represent a feedforward mechanism by which spinal dynorphin could maintain an elevated level of sensory excitation, whereas the potentiation of NMDA receptors would greatly facilitate the responsiveness of spinal thalamic neurons to excitatory input *(Lay J. et al. 2001)*.

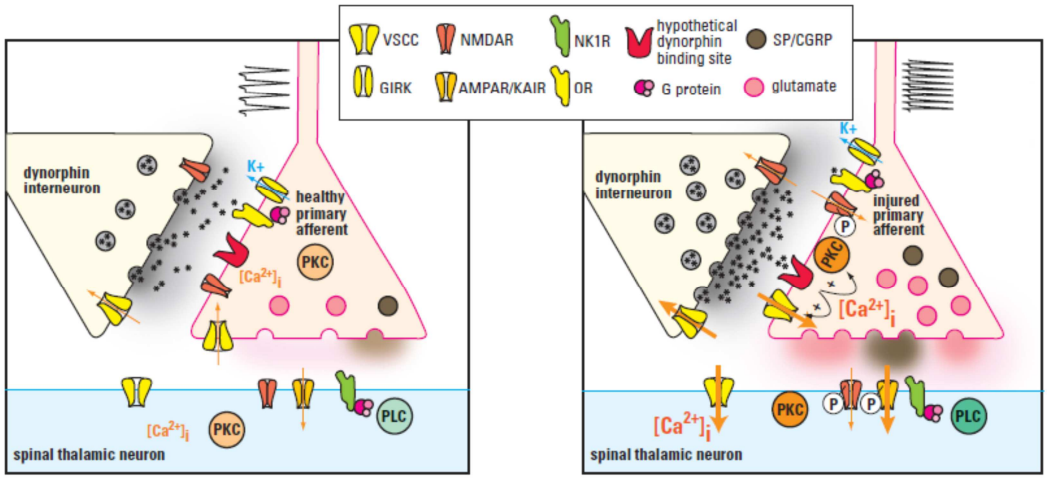


Figure 20 Possible mechanisms of dynorphin in maintaining neuropathic pain.

Under normal conditions (left panel), dynorphin acts at opioid receptors (OR) to limit noxious input from a primary afferent: Specifically, dynorphin activates potassium efflux (through a G protein–coupled inwardly rectifying potassium channel; GIRK) and inhibits calcium influx (through a voltage-sensitive calcium channel; VSCC) in primary afferent and spinal thalamic neurons. Thus, excitatory neurotransmitter (glutamate) release is inhibited from the primary afferent terminal. After nerve injury (right panel), sustained input from the injured nerve drives the upregulation of spinal dynorphin through an unknown mechanism. The enhanced release of dynorphin stimulates the activity of molecules such as protein kinase C (PKC) and VSCC at the primary afferent to facilitate release of excitatory neurotransmitter and neuropeptides (e.g., substance P [SP] and CGRP). The binding site that mediates the effects of dynorphin is unknown. Enhanced neurotransmission potentiates the activity of postsynaptic glutamate receptors (AMPAR/KAIR) and NMDA receptors (NMDAR) by calcium/PKC dependent receptor phosphorylation. This is in part due to the activation of the substance P receptor (NK1R)/phospholipase C (PLC) pathway *(Lay J. et al. 2001)*.

1.4 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a 27-kDa polypeptide that belongs to the neurotrophin family binding with high-affinity protein kinase receptors (Trk), especially TrkB, and the unselective p75NGFR receptor. The BDNF gene has a complex structure with multiple regulatory elements and four promoters that are differentially expressed in central or peripheral tissue *(Tapia-Arancibia L. et al. 2004)*. It is well known to play an important role in the survival, differentiation, and outgrowth of select peripheral and central neurons during development and in adulthood *(McAllister A.K. et al. 1999; Sohrabji F., Lewis D.K. 2006)*.

BDNF supports the survival and maintenance of sensory neurons, retinal ganglia, certain cholinergic neurons, spinal motor neurons and some dopaminergic neurons. In the brain, the synthesis of BDNF, as investigated in the hippocampus and cortex, is affected by neuronal activity and has a unique role in synaptic plasticity. The use or disuse of synaptic pathways results in strengthening or weakening of connections between neurons, resulting in increased or decreased formation of synapses at axon collaterals and dendritic spines *(Allen S.J. et al. 2013)*. The BDNF gene is one of the most tightly regulated genes, with 11 exons and 9 functional promoters *(Pruunsild P. et al 2007)* and gives rise to approximately 34 transcripts *(Gabriele B., TongiorgiE. 2009)* in response to various stimuli. Importantly, whereas the other neurotrophins are released via the constitutive pathway, BDNF can also be secreted via an activity-dependent regulated secretory pathway in neuronal cells. Processing by proBDNF or proNGF to themature form may occur inside or outside the cell. Intracellular processing is via furin; extracellular processing occurs via plasmin, generated following the activation of plasminogen by tissue plasminogen activator (tPA), which is co-released with proBDNF *(Pang P.T. et al. 2004)*. During regulated secretion, BDNF (the mature domain) associates with a sorting receptor carboxypeptidase E (CPE), whilst the pro domain binds the sortilin receptor ectodomain *(Zhang C.F. et al. 1999)*. ProBDNF also associates with the huntingtin-associated protein-1 (HAP1); sortilin stabilises the complex of proBDNF and HAP1, thus reducing proBDNF degradation. This also assists in the intracellular cleavage of proBDNF by furin to produce mature BDNF, and release by the secretory pathway

(Yang M. et al. 2011). A common polymorphism (proBDNFVal66Met) precludes the binding of proBDNF to sortilin which is thus not able to undergo regulated secretion. There are a number of consequences to this, one of which may be impairment of memory function *(Egan M.F. et al. 2003).*

1.4.1 Trk receptors

The Trk family of receptors includes TrkA, TrkB and TrkC which are instrumental in carrying out the cellular effects of neurotrophins. P75NTR, a member of the tumour necrosis receptor superfamily exhibits some structural similarities to Trk family of receptors and interacts with members of the Trk family including TrkB receptor and influences its signalling. The Trk receptors are a major class of neurotrophin receptors, as they promote cell survival and inhibit apoptosis in the specific populations of CNS neurons. Trk receptor signaling is activated in response to neurotrophin binding; each neurotrophin has high affinity for a particular receptor, Nerve Growth Factor (NGF) binds to TrkA, Brain-Derived Neurotrophic Factor (BDNF) and NT-4/5 bind to TrkB and NT-3 binds TrkC *(Skaper S.D. 2012).* (Fig. 21)

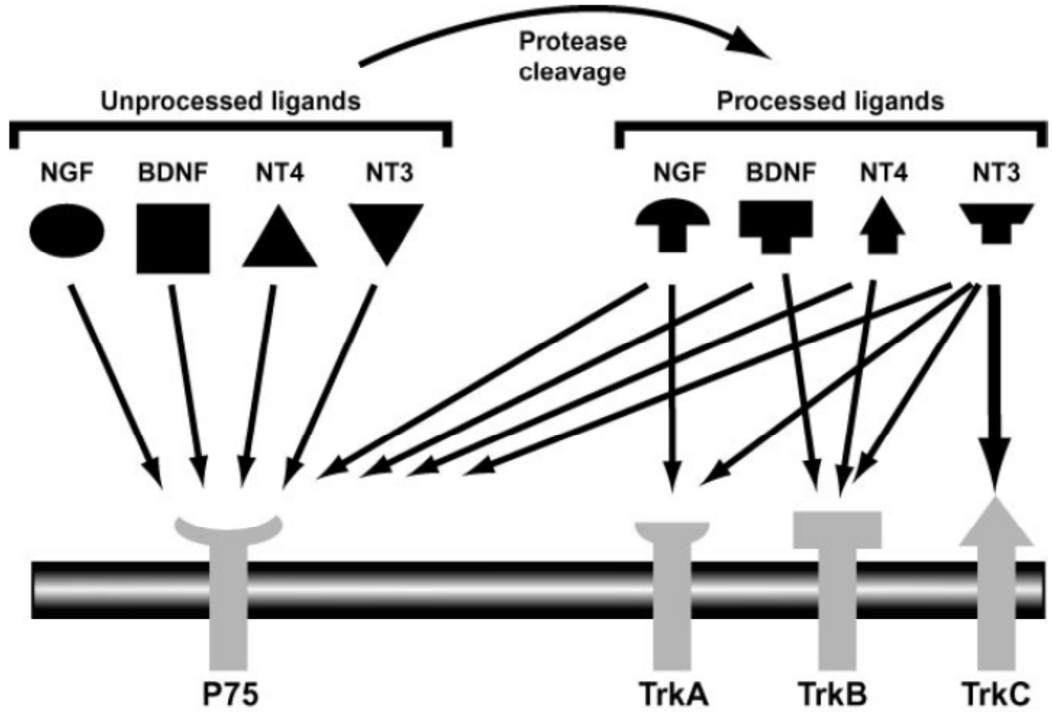


Figure 21 Neurotrophins and their receptors.

Neurotrophins are synthesized then processed by protease cleavage to generate the mature, processed ligands. All the unprocessed neurotrophins bind with high affinity to the p75NTR; the processed isoforms also bind to p75NTR, albeit with lower affinity. The processed ligands bind to the Trk receptors, NGF binds TrkA and BDNF, NT4 binds TrkB, and NT3 preferentially binds to TrkC but can activate all the Trks *(Segal R.A. 2003).*

The three Trk receptors exist as monomers and their dimerization is induced by the binding of their respective dimeric neurotrophins (Fig. 22). Specifically, BDNF associates with the extracellular domain of TrkB by interacting with the third leucine rich motif, the cysteine cluster-2 domain and the immunoglobin-2 domain, thereby stimulating receptor dimerization. Upon BDNF binding, TrkB receptor is autophosphorylated in the activation loop, of its cytoplasmic tyrosine kinase domain, at Tyr702, 706 and 707 (TrkA-Tyr670, 674 and 675), which enhances subsequent phosphorylation at two additional residues, which will act as docking sites for downstream effectors. Phosphorylation of TrkB-Tyr516 (or TrkA Tyr490) induces an interaction between the receptor and the PTB domain of Shc, which initiates Ras-MAPK and PI3K signaling pathways and subsequently, activates Akt1/PKB. Similarly, phosphorylation of TrkB-Tyr817 (or TrkA Tyr785) facilitates association between the receptor and PLCγ1, which stimulates the release of Ca^{2+} and upregulates PKCδ *(Hubbard S.R. 2007).*

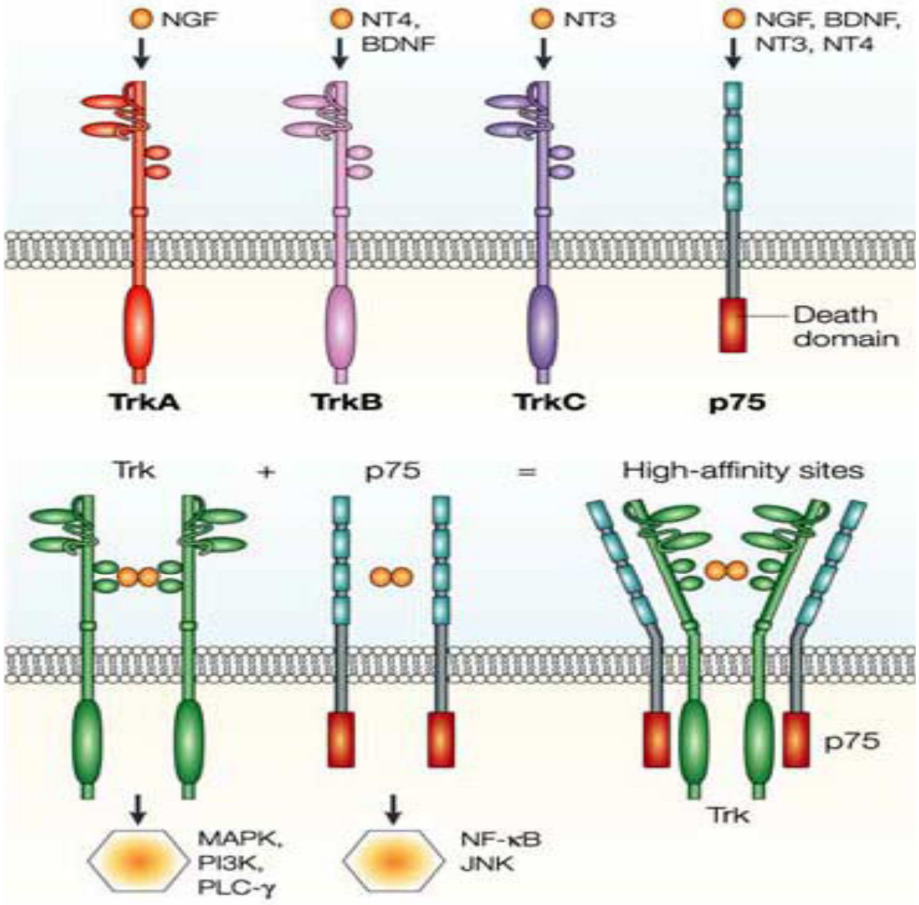


Figure 22 The binding of neurotrophins produces dimerization of each receptor.

1.4.2 BDNF and neuropathic pain

In experimental animals, peripheral nerve damage, such as that generated by chronic constriction injury (CCI) of the sciatic nerve, induces pain-related behaviours that are accepted as a model for human neuropathic pain. Seven or more days of CCI promotes release of cytokines, chemokines and neurotrophins at the site of nerve injury. These interact with first order primary afferent neurons to produce an enduring increase in their excitability. The central terminals of these damaged afferents exhibit spontaneous activity and release additional cytokines, chemokines, neuropeptides, as well as ATP and brain derived neurotrophic factor (BDNF) into the dorsal horn. These exert long term effects on dorsal horn excitability and/or alter the state of activation of spinal microglial cells. Microglia stimulated in this way release of a further set of mediators, again including BDNF, that promote a slowly developing increase in excitability of second order sensory neurons in the dorsal horn of the spinal cord (Fig. 23). This 'central sensitization' which develops progressively during CCI, is responsible for the allodynia, hyperalgesia and causalgia that characterize human neuropathic pain *(Biggs J.E. et al 2010).*

In CCI model, NGF is produced abundantly around degenerating Ab and Ad fibers by Schwann cells and macrophages. This NGF is retrogradely transported by intact C fibers to the DRG, leading to an increased production of BDNF in small-to-medium neurons. Corroborating the hypothesis of changed target-derived NGF as cause of altered BDNF expression, *Obata K. et al. 2004* administered recombinant NGF intrathecally (i.t.) in Sprague–Dawley rats and found an increased BDNF expression in small and medium DRG neurons. An increasing number of studies indicate that pain is not solely mediated by neurons but that glial cells and astrocytes have an active role in the initiation and maintenance of neuropathic pain. A way in which microglia may contribute to neuropathic pain is through the activation of their P2X4 receptor by ATP. This leads to an influx of extracellular calcium and subsequent activation of p38 MAPK resulting in an increased synthesis and release of BDNF through soluble N-ethylmaleimide-sensitive factor attachment protein receptor dependent exocytosis *(Vanelderen P. et al. 2010).*

Whereas microglial activation triggers pain onset, enduring activation of astrocytes is thought to be responsible for the maintenance of central sensitization. Changes in thalamic and cortical physiology, long-term sensitization of peripheral nociceptors and changes in descending inhibition from the rostral ventromedial medulla and periaqueductal grey and are also involved.

Despite the documented importance of interleukin 1β (IL-1β) and tumor necrosis factor a (TNF-α), MCP-1/CCL-2, ATP, BDNF and fractalkine in central sensitization, findings to be reviewed below point to the possibility that BDNF is alone capable of bringing about one critical step; the interaction between activated microglia and neurons. It may therefore serve as a final common path for a convergence of perturbations that culminate in the generation of neuropathic pain (Fig. 23). Several lines of evidence are consistent with the central role for BDNF in the initiation of central sensitization. BDNF released by ATP stimulated microglia causes a depolarising shift in the anion reversal potential of lamina I neurons by ways of a reduction in the expression of the potassium-chloride exporter KCC2. The resultant perturbation of the chloride gradient leads to attenuation of the inhibitory actions of GABA/glycine. In some neurons, the chloride gradient may actually reverse so that inhibition is converted to excitation *(Biggs J.E. et al 2010, Coull J.A. et al. 2005).*

Another possible mechanism by which BDNF induces central hypersensitivity is by enhancing NMDA receptor-mediated responses in the DH: the ventral root potential is routinely used as an indirect but accurate measure of spinal excitability. NMDA evoked ventral root depolarization amplitudes increased significantly after BDNF superinfusion in isolated hemisected spinal cords *(Vanelderen P. et al. 2010).* All these observations suggest a pro-nociceptive effect of BDNF, at least at the level of the spinal cord. Although it has been reported that viral vector-driven expression of BDNF and grafting BDNF-expressing cells into the spinal cord reduces signs of pain associated with CCI this may reflect analgesic actions within the midbrain.

These and other observations raise the possibility that BDNF is alone capable of conveying many aspects of the communication between activated microglia and neurons during the onset of central sensitization. This appears to occur despite the presence and potential participation of mediators such as IL-1β, TNF-α, fractalkine,

chemotaxic cytokine ligand 2 also known as monocyte chemoattractant protein 1 (CCL-2/MCP-1). These may exert their actions at other points in the central sensitization process (Fig. 23) or perhaps function in a parallel fashion to BDNF in microglial - neuron interactions *(Biggs J.E. et al 2010).*

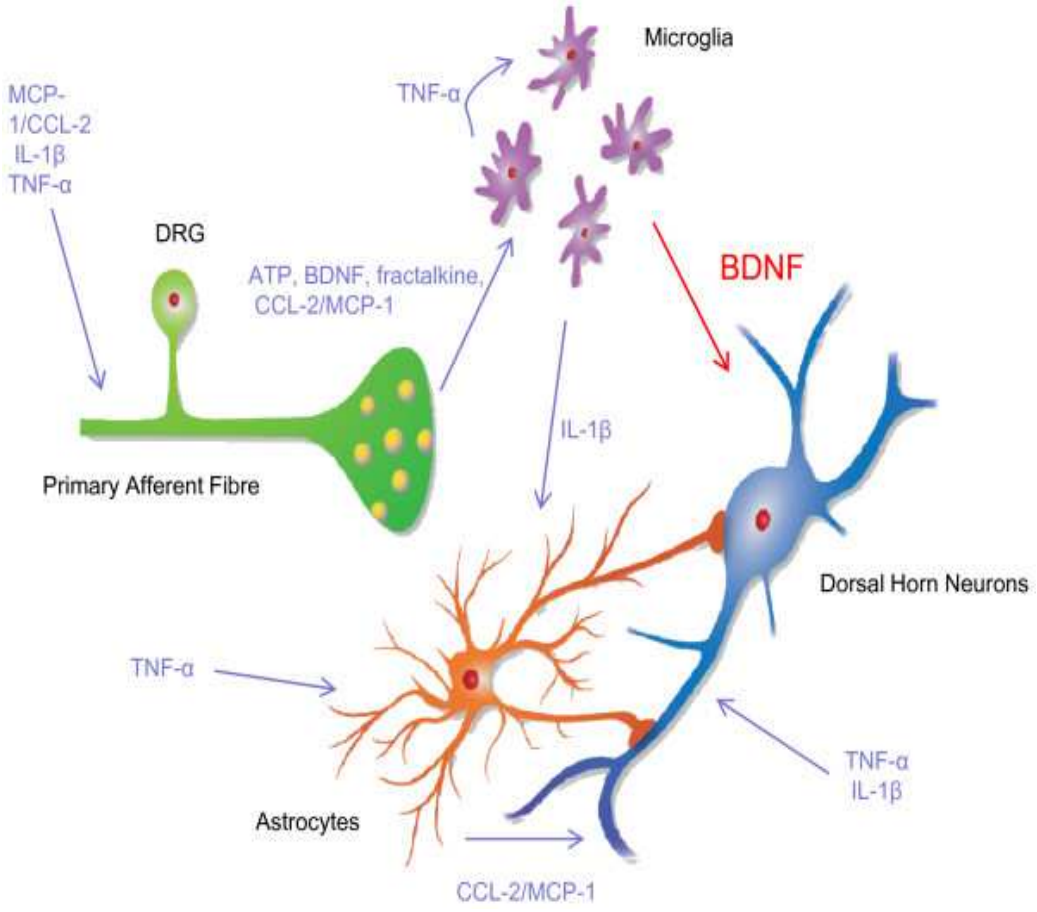


Figure 23 Scheme to show interactions between primary afferents, dorsal horn neurons microglia and astrocytes in the context of chronic pain.

Literature citations supporting the illustrated interactions include; IL-1β, MCP-1/CCL-2 and TNF-a in acute and chronic excitation of primary afferents; MCP-1/CCL-2, ATP, BDNF and fractalkine in microglial activation; autocrine actions of TNF-α in microglia; IL-1β release from microglia and its actions on neurons; BDNF release from microglia and its actions on neurons; role of MCP-1/CCL-2 in astrocyte-neuron interactions, actions of TNF-α on astrocytes and neurons. To the best of our knowledge actions of IL-1β on astrocytes in spinal cord has not been demonstated but there is evidence for this interaction in other neuronal systems *(Biggs J.E. et al 2010).*

1.5 Epigenetics

1.5.1 General consideration

Epigenetics (from the Greek, epi-: *επί*- over, above; and -genetics) correspond to the study of heritable changes in gene expression or in the cellular phenotype caused by mechanisms other than changes in the DNA sequence *(Feinberg A.P., Tycko B. 2004)*. Cellular differentiation is a well know example of relevance of epigenetic mechanism. If all cells within an organism have the same DNA *(Nestler E.J 2009)* then the ability to have different cells with different functions must be due to a selective activation or silencing of particular genes within genome *(Grewal S.I., Moazed D. 2003)*. Actually, it has been demonstrated that epigenetic events, altogether with genetic events, plays a crucial role in tumor progression *(Jordà M., Peinado M.A. 2010)*.

Three epigenetic mechanisms are considered the most important ones: genomic imprinting, histone modifications and DNA Methylation *(Feinberg A.P., Tycko B. 2004)* (Fig. 24). Genomic Imprinting refers to the relative silencing of one parental allele compared with the other parental allele as consequence of differentially methylated regions within or near imprinted genes.

Histone modifications, principally acetylation, methylation and phosphorylation, are important in transcriptional regulation due the ability to induce chromatin structure modification, altering DNA accessibility *(Feinberg A.P., Tycko B. 2004)*. DNA methylation is the most common epigenetic mechanism *(Jordà M., Peinado M.A. 2010)* and consists in a covalent modification of DNA, in which a methyl group is transferred from S-adenosylmethionine to the C-5 position of cytosine by a family of cytosine (DNA-5)-methyltransferases *(Feinberg A.P., Tycko B. 2004)* and occurs predominantly in the cytosines that precede guanines (CpG) *(Bird A.P. 1986)* (Fig. 25).

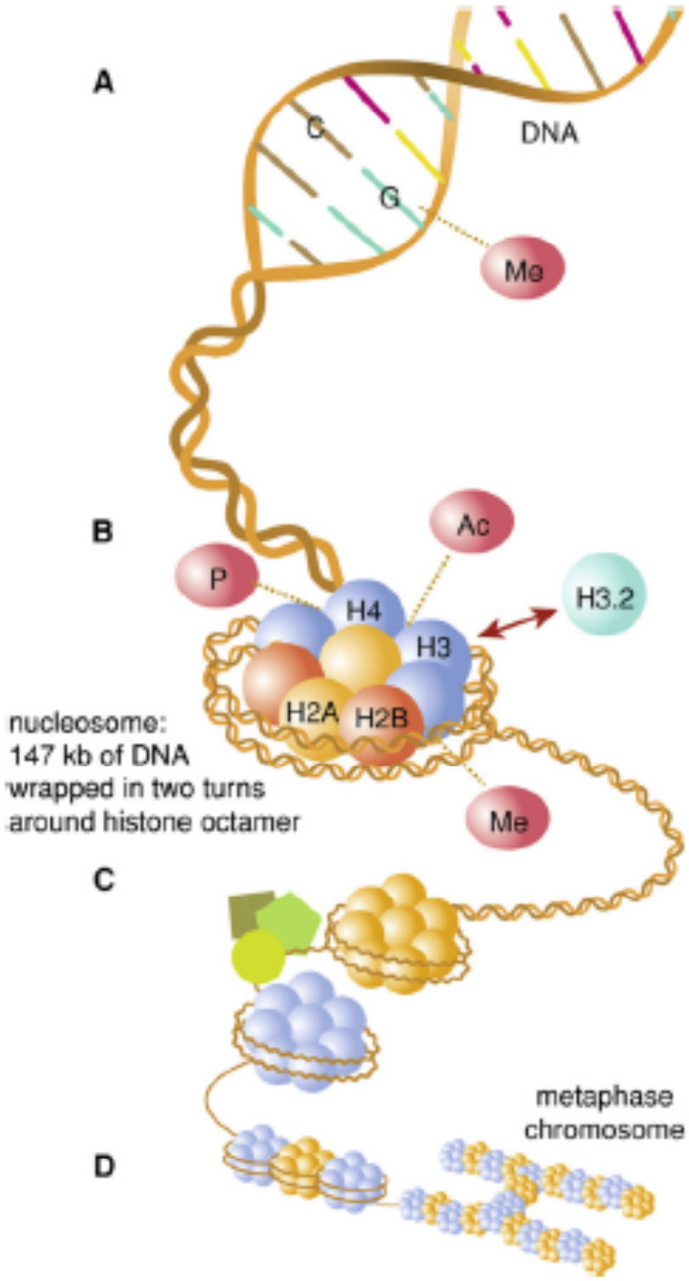


Figure 24. Schematic Representation of Epigenetic Processes and General Chromatin Structure
(A) DNA methylation. (B) Histone variants and modifications. (C) Chromatin remodeling complexes. (D) Nucleosomes.

1.5.2 Histone modifications

Gene expression can also be modulated by the chromatin state. DNA is packed in the nucleus of eukaryotic cells through its chromatin organization. The nucleosome, the fundamental unit of chromatin structure, consists of 146 base pairs of DNA wrappped around an octamer of histone made up of two copies of each of the core histone (H2A, H2B, H3 and H4) *(Kouzarides T. 2007)* (Fig. 25).

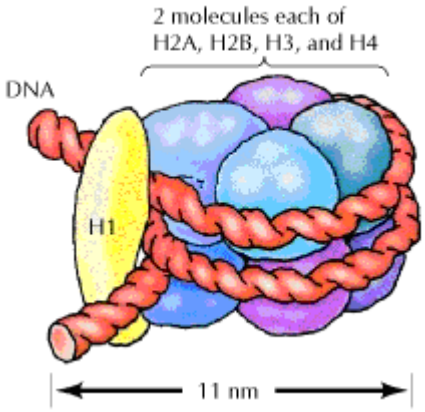


Figure 25. DNA wrapped around histone octamers to form nucleosomes

Each core histone is composed of a structured domain and an unstructured amino-terminal tail of varying lengths from 16 amino acid residues for H2A, 32 for H2B, 44 for H3 and 26 for H4, protruding outward from the nucleosome *(Taniura H. et al. 2007).* These proteins provide not only a solid structure; N-terminal regions of histones which protrude from the nucleosome are susceptible to interactions with other proteins. Chromatin can exist either in a decondensated, active arrangement, termed euchromatin, or in a condensated, inactive state, i.e. heterochromatin.

The post-translational modification of the residues at histone tails are: methylation of lysines and arginines, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. Two widely studied histone modifications are histone acetylation and phosphorylation.

Histone acetylation is linked with transcriptional activation, while deacetylation is related to transcriptional repression *(Berger S.L. 2007).*

Histone acetylation is a reversible modification of lysine residues within the amino-terminal tail domain of core histone; histone acetyltransferase (HATs) transfers an acetyl-group from acetylcoenzyme A to the ε-amino group of the lysine residue, while histone deacetylase (HDACs) acts in the reverse to remove the acetyl group. Also histone can be methylated by histone methyltransferases, inducing changes in the chromatin structure.

Methylation may create binding sites for other regulatory proteins thus influencing the chromatin structure, either condensating or relaxing the structure *(Chouliaras L. et al. 2010).*

Although DNA methylation and histone modifications can act independently, they can also interact with each other. DNA methylation is associated with histone modifications through methyl CpG binding proteins interaction with dynamic complexes containing histone-modifying enzymes that promote gene repression and DNA replication and repair *(Klose J., Bird A.P. 2006).*

The binding of some deoxy-methylcytosine binding proteins to methylated sequences attracts complexes containing co-repressors and histone deacetylases, leading to a change in the chromatin structure from an open, trancriptionally active form to a more compact, inactive form, inaccessible to the transcription machinery *(Richardson B. 2003).*

1.5.3 DNA Methylation

DNA methylation appears to be one of the most important epigenetic mechanisms used by the cell, for the establishment and manteinance of the correct patterns of gene expression *(Egger G. et al. 2004).*

DNA methylation patterns are stablished during differentiation, and serve to suppress genes unnecessary for the function of the mature cell. DNA methylation in mammals occurs in the cytosin of the CpG dinucleotide via a reaction catalysed by enzymes named DNA methyltransferases (DNMTs) and the recognition of methylated

cytosines is done by proteins that posses a specific binding domain, the so-called methyl-CpG binding domain. DNMTs are expressed throughout neural development, and in the adult brain in selective regional and cell-specific patterns including mature stem cell generative zones mediating ongoing neurogenesis *(Feng J. et al. 2007).*

Moreover, DNMTs are actively regulated by physiological and pathological states and interactions, and they promote neuronal survival, plasticity and stress responses *(Ooi S.K. et al, 2007).*

The methylation of CG sequences can affect nearby gene expression. Hypomethylation of regulatory sequences usually correlates with gene expression, while methylation results in transcriptional suppressor. In general, the more CpG islands located in the promoter of a gene, the more the trancription level is dependent on DNA methylation *(Graff J. Mansuy I.M. 2008)*.

Methylation of CpG units disrupts the binding of transcription factors and attracts proteins known as methyl-CpG binding domain proteins that are associated with gene silencing and chromatin compaction *(Antequera F., Bird A. 1993).*

The CpG islands, regions with more than 500 bp and a G + C content larger than 55%, are localized in the promoter regions of 40% of all the genes in mammals and are normaly maintaind in the non-methylated form *(Bird A.P., Wolffe A.P. 1999),* but the CpGs located outside the CpG islands are usually methylated *(Urdinguio R.G. et al, 2009).*

The importance of DNA methylation in the function of normal cells is evidenced by its role in differentiation, X chromosome inactivation, genomic imprinting maintenance of chromatine structure, and suppression of "parasitic" DNA.

It has been observed that multiple exogenous agents can affect DNA methylation, and it is possible that transient exposure to a DNA methylation inhibitor can have long term effects on DNA methylation.

1.5.4 Epigenetc and Pain

Evidence suggests that more than 1,000 genes in spinal dorsal horn neurons are epigenetically regulated within the first minutes to hours following a peripheral nerve injury *(Price D.D. et al. 2006; Tajerian M. et al. 2011; McCall C.E. et al. 2011).* Often, these early modifications are followed by more sustained epigenetic processes shaping synaptic connectivity and formation of pathologic long-term pain.

Several critical "first responder" transcription factors, including NF-kB (nuclear factor k light-chain-enhancer of activated B cells), c-Jun, c-Fos, and several hormone activated receptor proteins, serve as drivers for wide-ranging epigenetic responses to cellular stress. Present but inactive in many vascular, nerve, and immune cells, these transcription factors become activated in response to cell insult and are able to rapidly access chromosomal DNA as demethylation unspools chromatin structure to initiate production of a reparative cascade of inflammatory cytokines including TNF-α as well as T-cell and Bcell regulating interleukins. The nearly simultaneous dynamic remodeling of chromatin through the addition of methyl groups toDNA and the removal of acetyl groups from histone proteins regulates production of immune suppressing glucocorticoids, providing a critical check and balance to overactivation of immune responses. Epigenetic changes to chromatin structure are similarly linked to suppression of pain inhibiting GABA synthesis, changes in expression patterns of sodium and potassium channels driving afferent input into the spinal cord, and activity-dependent upregulation of pronociceptive brain-derived neurotrophin factor (BDNF) in the spinal cord, as well as functional regulation of mu opioid receptors, the principle receptor for endogenous endorphins, encephalin and as well as opioid analgesics.

Sustained DNA methylation downstream from early effector transcription factors, for example, has been linked to an accelerated degeneration of vertebral disks in low-back pain in both animal models and human subjects. Sustained histone deacetylation has been identified as a factor driving long-lived C-fiber dysfunction, decreased responsiveness to morphine analgesia, and an upregulation of pronociceptive metabotropic glutamine receptors in animal nerve injury models. Epigenetics processes also provide a mechanistic understanding of the phenomenon

of opioid-induced hyperalgesia, with chronic opioid use reported to stimulate DNA methylation leading to upregulation of μ-opioid receptors and increased pain with continued opioid use *(Lessans S., Dorsey G. 2013).*

2. AIM OF RESEARCH

The research project conducted during these years, is focused on the neuroplastic alterations occurring in chronic pain conditions.

Within this context, our attention has focused on the study of pathophysiological phenomena associated with nerve-injured neuropathic pain.

Chronic pain is an enormous medical-health problem, it is associated with a significantly reduced quality of life and a higher risk of depression and other mental health disorders. It was estimated that approximately 20% of the adult population have chronic pain, and separate to the physical and emotional burden it brings, the financial cost to society is huge, currently estimated at over €200 billion per annum in Europe, and $150 billion per annum in the USA *(Tracey I., Bushnell M.C. 2009).* Treatment options are limited with many patients either not responding or having incomplete pain reduction.

Neuropathic pain is not a single entity; it is a heterogeneous group of conditions that differs not only in aetiology but also in location and symptoms do neither respect cause nor anatomical site. Diabetes, immune deficiencies, malignant diseases, traumatic and ischemic disorders may all give rise to neuropathic pain. The anatomical sites of lesions causing neuropathic pain are multiple: they can be located from the peripheral receptor to the highest cortical centers. The most common locations are the peripheral nerves, the plexus, dorsal nerve roots, the spinal cord and the brain. In spite of the diverse aetiology and topography, the clinical picture is in many cases surprisingly similar suggesting that pain in these many disorders share common mechanisms. Different studies have shown that a cascade of temporally related biological changes follow damage to the nervous system, which eventually results in a sensitization of neural elements involved in the processing of noxious information *(Costigan M., Woolf C.J. 2000; Woolf C.J., Salter M.W. 2000.).* Therefore pain is an expression of maladaptive plasticity within the nociceptive system, a series of structural and functional changes that constitute a neural disease state (*Costigan M. et al. 2009).* Although the significance of these molecular changes following nerve damage still are under exploration, they may represent a link that ties different neuropathic conditions together *(Jensen T.S. et al. 2001).* Hence, an

understanding of the dynamic events after nerve damage may be a key to understand hyperexcitability and to move treatments from merely suppressing symptoms to a disease modifying strategy aimed at preventing this maladaptive plasticity.

Animal models provide pivotal systems for preclinical studies of neuropathic pain and serve as an experimental basis for mechanistic investigations and testing new therapeutic interventions. Over time, various tests and models were developed in rodents to provide tools for fundamental and translational research on the topic.

The ideal models should result in reproducible sensory deficits such as allodynia, hyperalgesia and over a sustained period. Animal research must always be evaluated by three general criteria: the generation of knowledge, the ability of the study to be reproduced, the relevance of the study and the predictive validity of clinical pain states. One of the most widely used experimental models, which more closely resembles behavior of peripheral neuropathy in rodents, is the chronic constriction injury to the sciatic nerve (CCI). It is reliable and easily reproducible model, able to induce hyperalgesia and allodynia (*Colleoni M., Sacerdote P. 2010*).

Through the use of this model it is possible to study molecular changes of different endogenous systems widely recognized to be involved in nociception.

The endogenous opioid system is probably the most important system involved in control of nociceptive transmission and it is therefore an important target of investigation in pain research. In this context, the neuropeptide dynorphin, though representing the endogenous ligand of the κ opioid receptor *(Chavkin C. et al. 1982)*, has been also suggested as a key mediator of some aspects of chronic/neuropathic pain by numerous studies *(Lai J. et al. 2001).*

Beside these system, an important role for certain neurotransmitters / neuromodulators such as BDNF has also been recently suggested. In fact, its involvement in the peripheral and central sensitization phenomena is known. At this regard, its involvement in the disinhibition caused by an alteration of the Cl^- cell gradient followed by the inversion of GABAergic inhibitory activity *(Kohno T. et al. 2005)* and by an increase in the NMDA receptors-mediated excitatory activity *(Vanelderen P. et al. 2010)* has been already reported in the Introduction.

Moreover, a possible link between BDNF and dynorphin has been recently proposed with a role of a downstream effector played by dynorphin in some actions involving BDNF *(Logrip M.L. et al. 2008).*

Beside the classical opioid receptors, a new GPCR receptor, initially called ORL-1 (opioid receptor like 1) and now known as NOP, has been identified, followed by the identification of its endogenous ligand: the neuropeptide nociceptin. This endogenous system, although structurally very similar to the classical opioid system, represent an independent system. It is well established that the nociceptin and all exogenous NOP receptor ligands have pronociceptive and antinociceptive effects depending on the drug dose and site of administration. If administered at a supraspinal level, the nociceptin induces hyperalgesia. Intrathecal and peripheral administrations cause hyperalgesia at low doses and analgesia at high doses *(Mika J. et al. 2010).*

Based on these evidences, the primary aim was to evaluate the effects on gene expression of precursors of opioid peptides and receptors (ppN/OFQ, pDYN, pENK, pNOP) and pBDNF at spinal level (SC and DRGs) at different intervals after nerve injury and to understand the physiological function of these systems in the development of neuropathic pain.

Based on results derived from studies at spinal levels and even more on the growing importance conferred on the cerebral neuroplastic alterations we investigated in the same experimental conditions gene expression changes at supraspinal level, focusing our attention on N/OFQ-NOPr system, DYN-KOPr system and BDNF.

Recently, an increasing number of experimental evidence suggest that changes in gene expression induced by pain condition may be mediated by epigenetic mechanisms *(McMahon S.B., Denk F. 2012)*. There are two types of chromatin modifications involved in the regulation of gene transcription: histone modifications and DNA methylation. Increasing evidences on the relationships between changes in methylation of lysine on histone 3, and alteration of gene expression are evident *(Boggs B.A. et al. 2002).*

Epigenetic mechanisms seem to play a role in neurol plasticity resulting from chronic pain *(Chwang W.B. et al. 2007; Koshibu K. et al. 2009; Lubin F.D. et al. 2008).*

With this purpose, studies have been performed using epigenetic Chromatin Immuno Precipitation assays (ChIP) in order to observe different changes in the promoter regions of DYN and BDNF genes, related to the alterations in gene expression, observed at spinal level.

We also investigated BDNF involvement in neuropathic pain through the analysis of the behavioral signs of hyperalgesia and allodynia in a strain of mice partially lacking for BDNF, subjected to CCI.

The interest on the N/OFQ-NOPr system has been further analyzed, in the constant experimental context, evaluating if levels of bioactive N/OFQ peptide could be related to gene expression changes observed in the same cerebral areas.

Simirarly, in the last part of the present study, in order to provide a unique approach to explore receptor localization and function *in vivo* of the N/OFQ-NOPr system, I focused my study on the characterization and validation of an innovative knock-in mouse strain. This model can be useful for a better characterization of pathophysiological role of this system in neuropathic pain.

In 2005, Kieffer and colleagues produced the first transgenic mice containing a GPCR, the δ opioid receptor, with enhanced Green Fluorescent Protein (EGFP) fused on the C-terminal *(Scherrer G. et al. 2006)*. According to this strategy the fusion of EGFP to NOP receptors could provide a unique technology useful to answer different questions concerning the involvement of N/OFQ-NOPr system in chronic pain condition. NOP-EGFP mice will allow the visualization of receptors in the brain, spinal cord and DRGs. In addition, this murine model will permit to quantify changes in NOP receptor levels caused by different experimental conditions, such as neuropathic pain, either in basal condition or after different pharmacological treatments.

3. MATERIALS AND METHODS

3.1 Animals

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National (Ministry of Health) laws and policies (authorization no. 204/2008-B). Care was taken to minimize the number of experimental animals and to take measures to minimize their suffering.

Adult male Swiss mice (ICR/CD-1, Harlan Lab., Udine, Italy) or BDNF +/- and BDNF +/+ (provided by Dr Marco Canossa, University of Bologna) weighing 20–30 g at the beginning of the experiment were housed five per cage in standard Macrolon cages (Tecniplast Gazzada, Buguggiate, Italy) in a temperature- and humidity-controlled room with a constant 12-hour light/dark cycle (lights on at 7am). The mice were allowed to acclimatize for at least 1 week before the start of the experiments, during this time the mice were handled daily. Free access to standard lab chow and tap water were available ad libitum, except during behavioral experiments.

NOP-eGFP knock-in mice, obtained from Dr. Brigitte Kieffer (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Ilkirch, France), were bred inthe Neuropharmacology Laboratory, directed by Dr. Lawrence Toll, at Torrey Pines Institute for Molecular Studies, Port St. Lucie, Fl, USA.

3.1.1 Transgenic BDNF mice

In the gene-targeting construct, a 560-bp fragment from the BDNF protein-coding exon was replaced by the selection marker-a neomycin-resistance gene flanked by a glycerate kinase gene promoter and a polyadenylylation signal-thus deleting most of the mature BDNF coding sequence (Fig. 26). Homozygous BDNF -/- mutant mice were retarded in growth and had reduced weight (down to only 25% of the wild type) from postnatal day 3 (P3) on. They displayed aberrant limb coordination and balance, showed a loss of neurons in the dorsal root ganglia, and usually died between 2 and 4 weeks after birth. Such abnormalities were never observed in heterozygous BDNF +/ - mice *(Korte M. et al. 1995).*

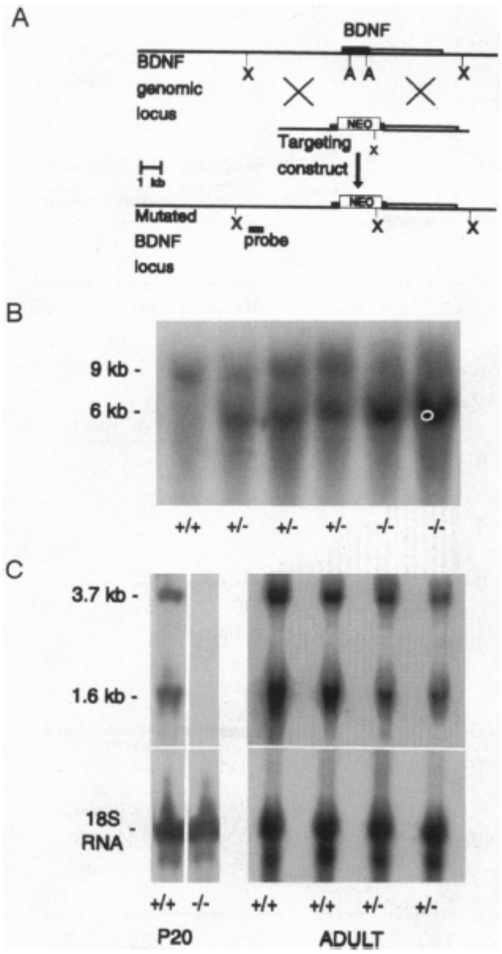


Figure 26 Production of BDNF-deficient mice.

3.1.2 NOP-EGFP mice

Knock-in mice were constructed so that the NOP receptor with eGFP fused at the C-terminus is expressed in place of the native receptor with eGFP attached to the C-terminal of NOP receptors. The mice grow and breed normally.

3.1.2.1 Genotyping.

Total DNA was isolated from the mouse tail using DNeasy Kit (Qiagen).
The NOP primers were:
5′-CCCTGCACCGGGAGATGCA-3′ (forward)
5′-GACAGAGGCCATGGAGGCC-3′ (reverse),
producing a 319 bp PCR product to amplify wild type NOP DNA.
The NOP-eGFP primers were:
5′-CCCTGCACCGGGAGATGCA-3′ (forward)
5′-GCGGACTGGGTGCTCAGGTA-3′ (reverse),
producing a 733 bp PCR product to amplify the NOP-eGFP transgenic DNA. PCR will be performed with an annealing temperature of 60°C using the GoTaq FlexiDNA kit (Promega). Fig.27

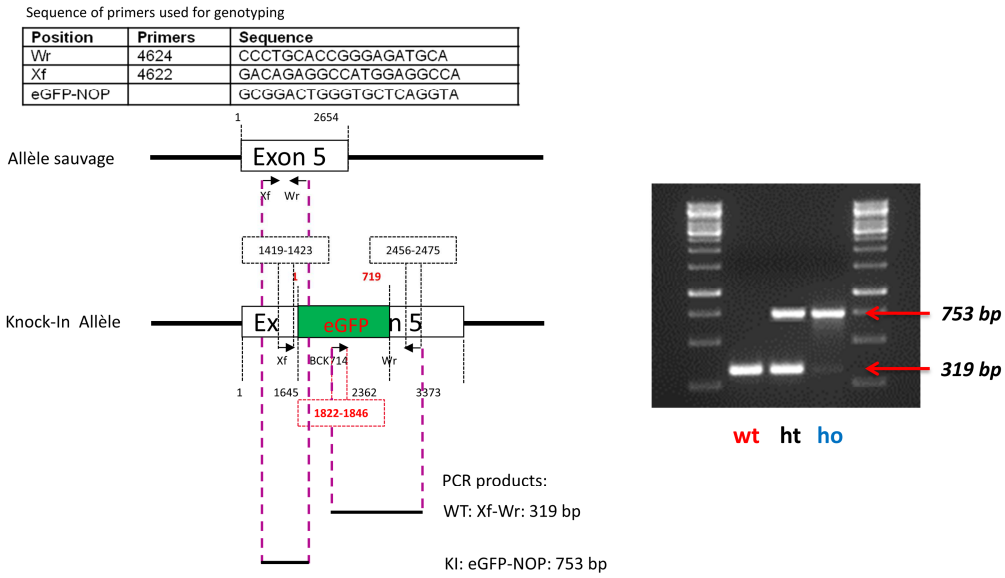


Figure 27 NOP-EGFP knockin mice. Targeting strategy and genotyping PCR products.

3.2 Surgical procedures

Mice were subjected to a right sciatic nerve lesion, according to the chronic constriction injury (CCI) model *(Bennet G.J, Xie Y.K. 1986)*, adapted to the mouse. Briefly, mice were anesthetized with (equitesina:Na-penthobarbital and chloral hydrate mixture; 30 mg/kg i.p.) and the lower back and right thigh were shaved. The shaved area was then cleansed with 70% ethyl alcohol. A linear skin incision was made along the lateral surface of the biceps femoris and blunt forceps were inserted into the muscle belly to split the muscle fibers and expose the sciatic nerve. The tips of the forceps were passed gently under the sciatic nerve and lifted to pass three 4-0 silk sutures under the nerve, 1mmapart. The sutures were then tied loosely around the nerve and knotted twice to prevent slippage. The incision was cleansed and the skin was closet. The mice were then placed on a warmed surface and following recovery, were returned to their home cages and checked routinely for 72 h. A separate control group of sham operated mice underwent the same surgical procedure with the exception of the ligation of the sciatic nerve.

3.3 Behavioral tests

The presence and development of behavioural signs of neuropathic pain were assessed by the Hargreaves' plantar test (hyperalgesia) *(Hargreaves K. et al. 1988)* and the cold acetone test or cold plate test (allodynia), performed at different intervals, three seven and fourtheen days, after the sciatic nerve lesion.

3.3.1 Plantar test

Paw withdrawal latencies to heat were measured according to the method of Hargreaves applying a standard Ugo Basile Algesiometer (Comerio, Italy). Animals were placed in the examination room 10 min prior to testing to allow acclimation to the light and testing environment. After 5 min, animals were placed in Plexiglas

enclosures with glass floors and a radiant heat source was positioned under one hind paw (Fig. 27). The intensity of the beam (30 A.I.) was selected to produce an average baseline threshold of approximately 10 s. A 30–second cut-off was employed to prevent tissue damage in non-responsive subjects.

The time to paw withdrawal was recorded automatically. Each hind paw was tested three times.

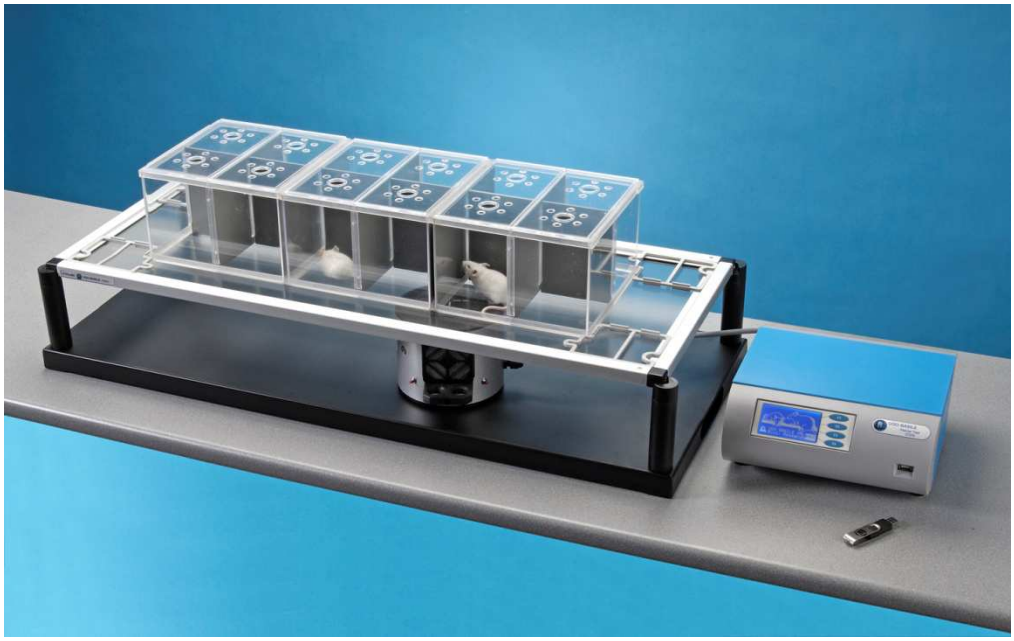


Figure 28 Plantar Test (Hargreaves's Apparatus); Ugo Basile

3.3.2 Acetone test

The acetone test was used to investigate paw response to cold. Mice were placed in plexiglass cages on a wire mesh. The plantar surface of the hind paw was touched with a drop (40 µl) of acetone and the reaction of the mouse was recoded, by assigning a score as shown in Tab. 4. The cut off time for the registration of the score was 30 seconds. Each hind paw was tested three times at 5-minute intervals to avoid sensitization *(Caspani O. et al. 2009).*

Score	***Behavior***
0	*No response*
0,5	*Licking response*
1	*Flinching and brushing of the paw*
2	*Strong flinching*
3	*Strong flinching and licking*

Table 4 Score Assignement Acetone test.

3.3.3 Cold-Plate test

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (*Bennett G.J, Xie Y.K. 1988)* (Fig. 28), as previously described *(Ruiz-Medina J. et al. 2011).* Briefly, mice were placed into compartment enclosures on the cold surface of the plate which is maintained at a temperature of 5 ± 0.5°C. Each animal was placed on the cold plate, and the time at which it showed a response, such as elevation of hindpaw, was recorded.

Figure 29 Cold Plate Test.

3.4 Tissue collection

Seven and Fourteen days after CCI, ICR-CD1 or BDNF +/- animals were sacrificed and the following areas were collected: the L4, L5 and L6 dorsal root ganglia (DRGs), the L4-L6 segment of the spinal cord (SC), the brainstem (BS), thalamus (Th), amygdale (Amy), hypothalamus (Hyp), hippocampus (Hippo), caudate-putamen (CP), nucleus accumbens (Nac) as well as the somatosensory (SSCx), prefrontal (PFCx), and anterior cingulate cortices (ACCx) and stored at a -80° C until use.

3.5 Real-Time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

3.5.1 Extraction of total RNA

Total RNA was prepared according to the method previously described *(Chomczynski P., Sacchi N. 1987)*. The RNA was extracted from single tissue samples by homogenizing with TRI Reagent solution (Ambion Inc. Italy), containing phenol and guanidine thiocyanate (Ambion), 1 mL TRI Reagent solution per 50-100 mg tissue. Then, 0.2 ml chloroform/2 ml of homogenate, and centrifuging the suspension at 12,000 x g for 10-15 minute at 4°C, and was transfered the aqueous phase to a fresh tube. A volume of 0.5 ml isopropanol was added, incubated for 15 min at 4°C and the RNA pellet was isolated by centrifugation at 12,000 x g for 25 min at 4°C.

The pellet was washed twice with 75% ethanol, dried under vacuum and then resuspended in 25 µl of Rnase-free water. Total RNA, digested with DNase RNase-free enzyme to eliminate genomic DNA content, was quantified by measurement of absorbance at 260 nm (1OD/ml = 40 µg RNA/ml). The ratio OD260/OD280 > 2 provided an estimate of the purity of the total RNA.

The quality of total RNA was evaluated by 1% agarose gel.

3.5.2 Reverse Transcription and PCR

RNA samples were subjected to DNase treatment and converted to cDNA with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) by using random hexamers (0.45 µg of total RNA in a final reaction volume of 20 µl). The cDNAs were subsequently diluted three times.

Relative abundance of each mRNA species was assessed by real-time RT-PCR employing 2 µl of the diluted samples in a final volume of 20 µl using SYBR Green MasterMix (Life Technology) on a StepOne Real Time PCR system (Life Technology, Applied Biosystem).

To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined and the point of early log phase of product accumulation is defined by assigning a fluorescence threshold above background defined as the threshold cycle number or Ct.

Differences in threshold cycle number were used to quantify the relative amount of PCR target contained within each tube. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2-DDCt) for statistical analysis *(Pfaffl M.W. 2001; Livak K.J, Schmittgen T.D 2001).*

All data were normalized to the endogenous reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Results on RNA were normalized to results obtained on RNA from the control, vehicle treated rats. After PCR, a dissociation curve (melting curve) was constructed in the range of 60 ºC to 95 ºC to evaluate the specificity of the amplification products. The primers used for PCR amplification (Tab. 5) were designed using Primer 3.

Primer	FORWARD (5'-3')	REVERSE (5'-3')
NOP	TCAGTTCATTGTGCTCCTGC	GCCATACAAGACCTCCCAGA
ppN/OFQ	TTTGTGACGTTCTGCTGCTC	GCATACAGTCCAGAGAGGGC
pDYN	CCCTCTAATGTTATGGCGGA	AGAGACCGTCAGGGTGAGAA
pENK	CTACAGGCGCGTTCTTCTCT	CAGCTGTCCTTCACATTCCA
pBDNF	GCGGCAGATAAAAAGACTGC	CCTATGAATCGCCAGCCAAT
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Table 5. Primer sequences used for reverse-transcription-polymerase chain reaction.

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; pKOP = kappa opioid receptor; pNOP = nociceptin opioid receptor; pDYN = prodynorphin; ppN/OFQ = prepronociceptin; pENK = proenkephalin; pBDNF = proBDNF

3.6 Epigenetic studies

Analysis of histones modification by Chromatin Immunoprecipitation (ChIP) and Real Time PCR

Chromatin was prepared from frozen tissues as previously described with minor modifications *(Dahl J.A., CollasP. 2007)*: proteins were cross-linked to DNA by addition of formaldehyde at a final concentration of 1% in phosphate buffer saline (PBS) containing a broad-range protease inhibitor cocktail (PIC) (Roche) and butyrate (Sigma), for 8 min at room temperature. The cross-linking reaction was quenched by adding glycine to a final concentration of 0.125 M and incubating for 5 min at room temperature, the sample washed, and lysed thorough resuspension by pipetting in 120 ul of lysis buffer.

The sample was incubated on ice and sonicated for 30 s to shear the DNA to fragments ranging in size from 150 to 700 bp, as analyzed by agarose gel electrophoresis.

The lysate was centrifuged at 12 000 g for 10 min at 4°C and the supernatant transferred into a chilled tube, leaving around 30 ul of buffer with the pellet. Another 30 ul of lysis buffer was added and the tube vortexed. After centrifugation as before, 50 ul of the supernatant was pooled with the first supernatant and sonicated for another 2 X 30 s on ice.

After removing a few µl to serve as "input" DNA, for each immunoprecipitation, 8 µg of chromatin was diluted 10-fold in RIPA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 140 mM NaCl) containing PIC and incubated overnight by rotation with either no antibody as control or with 1 - 4 µg of antibody, previously coated with Protein A beads (Invitrogen), for 2 hours at 4°C with agitation, against either H3K4me3 (Abcam, ab8580), H3K27me3 (Millipore, 17-622), H3K9Ac (Millipore, 17-658).

The beads and associated immune complexes were washed three times with RIPA buffer and once with Tris-EDTA buffer. The immune complexes were eluted with elution buffer (20 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl) containing proteinase K (50 µg/ml) at 68°C for 2 hours, and DNA was recovered by phenol extraction,

ethanol precipitated, and resuspended in 50 µl of sterile water. This procedure has been described in more detail *(Dahl J.A., Collas P. 2007)*. Thereafter, real-time qPCR (RT-qPCR) quantification of the genomic sequences from regions in the rat PDYN and PNOC proximal promoter associated with the immunoprecipitated proteins were carried out. The primers used for PCR amplification were designed using Primer 3 software (Tab. 6) *(Rozen S., Skaletsky H. 2000)*:

Primer	**FORWARD (5'-3')**	**REVERSE (5'-3')**
PrompDYN	GGCTTCCTTGTGCTTCAG	TAGCTGCTCCTGGTGATG
PrompBDNF	TTCGATTCACGCAGTTGTTC	CTGAGCCAGTTACGTGACCA

Table 6 Primer sequences used for Real Time PCR.

The relative abundance was assessed by RT-qPCR using SYBR Green MasterMix (Life Technology) on a StepOne Real Time PCR system (Life Technology, Applied Biosystem).

To provide precise quantification of the initial target in each PCR reaction, the amplification plot was examined and the point of early log phase of product accumulation defined by assigning a fluorescence threshold above background, defined as the threshold cycle number or Ct.

Differences in threshold cycle number were used to quantify the relative amount of the PCR targets contained within each tube. After PCR, a dissociation curve (melting curve) was constructed in the range of 60 ºC to 95 ºC (22) to evaluate the specificity of the amplification products. The relative expression of different transcripts was calculated by the delta-delta Ct (DDCt) method and converted to relative expression ratio (2-DDCt) for statistical analysis *(Livak K.J., Schmittgen T.D. 2001)*.

All ChIP data were normalized to the input DNA amounts (Ct values of immunoprecipitated samples were normalised to Ct values obtained from 'input'). In addition, results on DNA from treated samples were normalized to results obtained on DNA from the control sample. Each ChIP experiment was repeated at least three times.

3.7 N/OFQ levels determination

3.7.1 Extraction

Tissue samples (brain areas) were sonicated in 10 volumes (vol/wt) of boiling 1 M acetic acid, and maintained at 90°C for 10 minutes. After centrifugation (12,000 rpm for 20 min at 4°C), the supernatants were separated and subjected to chromatographic extraction as previously described (*Aparicio L.C. et al. 2004).*
Supernatants were mixed with an equal volume of TFA (1% v:v) and loaded onto previously equilibrated C 18 cartridges (SEPCOL- 1; Bachem, U.K.). Microcolumns were washed twice with 3 ml of 1% TFA and then eluted with 3 ml of 60% acetonitrile in 1% TFA. Eluates were lyophilized and stored at -80°C until RIA.

3.7.2 Radioimmunoassay

Immunoreactive N/OFQ (ir-N/OFQ) present in tissue extracts was measured by a specific RIA according to a validated procedure *(Ploj K. et al. 2000)*. Lyophilized extracts were reconstituted with 50 ml of methanol/0.1% HCl (1/1) and assayed in duplicate. Aliquots of 25 µl were mixed with 100 µl of 125I-N/OFQ (Bachem, St. Helens, Merseyside, UK) and 100 µl of antiserum (96:2+; kindly supplied by Prof. SC I. Nylander, Uppsala University, Sweden). The antiserum was used at the appropriate dilution to give 30%-34% binding of the [125I]- N/OFQ (4600e5000 cpm. The labeled peptide and the antiserum were diluted in a gelatin buffer containing 0.15 M NaCl, 0.02% sodium azide, 0.1% gelatin, 0.1% Triton X-100, and 0.1% BSA in a 0.05 M sodium phosphate buffer (pH 7.4). RIA tubes were incubated at 4°C for 24 hours. A charcoal slurry (1 ml/tube) was used to separate free and antibody-bound peptide (15% horse serum, 3% charcoal, 0.3% dextran in RIA buffer). Bound peptide was separated by centrifugation (5,000 3 g at 4°C), and 1 ml aliquots of the supernatants were counted for 1 minute on a Beckman 5,500 gamma counter (Beckman, Fullerton, CA). The detection limit of the RIA assay was 1–2 fmol/tube. RIA curves and data were analyzed using the GraphPad Prism 4 software for Windows (GraphPad Software, San Diego, CA).

3.8 Primary Cultures

Primary brain cultures derived basically as described by Scherrer et al, 2006. Briefly, Newborn NOP-EGFP mice pups (P0) were decapitated, and appropriate brain regions, removed. Tissue was digested by papain (15 Units/ml; Worthington). Cells were plated on poly-D-lysine coated coverslips at a density of 80,000 cells per cm2. Cells were grown in neurobasal A medium (Invitrogen) with B27 supplement, 0.5 mM glutamine, 5 ng/ml basic fibroblast growth factor (bFGF, Sigma), and antibiotics. Internalization studies were conducted 10-14 days after plating.

3.8.1 Time-Lapse Fluorescence Microscopy

Primary cells from NOP-EGFP mice were seeded in glass-bottom, 32-mm diameter plastic dishes coated with poly-D-lysine (Sigma). Fully matured primary neurons (10–14 days in vitro) were used, and receptor internalization studies were performed in the presence of N/OFQ. Samples were observed under a Delta Visione Elite Microscope with objective 60X at 37°C. Images were automatically recorded during 20 min, with increasing time intervals to avoid bleaching effects because of repetitive scanning. Specifically, 20 frames every 10 s followed by 10 frames every 30 s, and then 12 frames every minute were recorded, for a total of42 images.

3.9 Brain Slices.

6-8 week old NOP-EGFP mice were anaesthetized with isofluorane and transcardially perfused with PBS 1X followed by 4% paraformaldehyde. Brains were dissected, washed with a gradient of sucrose 10% 20% and 30%, plunged in Optimal Cutting Temperature (OCT) and stored at -80°C until sectioning. All brains were sectioned coronally on a Leica CM1850 cryostat at the thickness of 16 μm, then mounted on polylysine-coated microscope slides, stained with DAPI (Southern Biotech).

All samples were observed under Delta Vision Elite Microscope and the SoftWorx software was used for image acquisition.

3.10 Statistical Analysis

Hyperalgesia signs data were analyzed by Dunnett's test or the two tailed Student's *t*-test. Allodynic signs data from Acetone Test were analyzed non-parametrical analysis (*Mann–Whitney U-test*). Allodynic signs data from Cold Plate Test were statistically asnalyzed using the *one-way ANOVA* followed by *Newmann-Keuls*.
Gene expression data were statistically asnalyzed using the Dunnett's test or the two tailed *Student's t-test*.
Histone modification changes in SC and N/OFQ peptide levels alterations in different brain areas were statistically analyzed using the two-tailed *Student's t- test*. Statistical significance was set at $P < 0.05$.

4. RESULTS

4.1 Opioid System and BDNF alterations studies in ICR/CD1 neuropathic mice

4.1.1 Determination of the behavioral signs of neuropathic pain

After recording the baseline values for both CCI and sham groups, the development of thermal hyperalgesia and cold allodynia were assessed three, seven and fourteen days after surgery using respectively the Hargreaves's Plantar test and the Acetone Test. Sciatic nerve ligation caused a significant decrease of the paw withdrawal latency three days after surgery in the CCI animals compared to Sham group (4.111 ± 0.601 vs Sham 9.197 ± 1.931, unpaired t test $*P < 0.05$, $***P < 0.001$), and the thermal threshold of the CCI group remained lower at 7 and 14 days after sciatic nerve ligations compared to Sham animals (respectively, 2.910 ± 0.615 vs Sham 10.217 ± 1.316, 2.315 ± 0.381 vs Sham 10.507 ± 0.869, unpaired t test, $***P < 0.001$). (Fig. 30a). As shown in Fig. 30b, CCI animals exhibited no allodynic response to a cold stimulus (acetone) prior to ligation of the sciatic nerve; three days after surgery, there was an increase in the average response to acetone evaporation of the CCI animals compared to Sham group (0.708 ± 0.042 vs Sham 0.056 ± 0.035, unpaired t test, $***P < 0.001$). Seven days after ligation there was an enhanced increment in the average response of the CCI mice compared to Sham ones (2.340 ± 0.280 vs Sham 0.389 ± 0.176, unpaired t test, $***P < 0.001$), with comparable values fourtheen days after the lesion (2.233 ± 0.280 vs Sham 0.067 ± 0.041 unpaired t test, $***P < 0.001$).

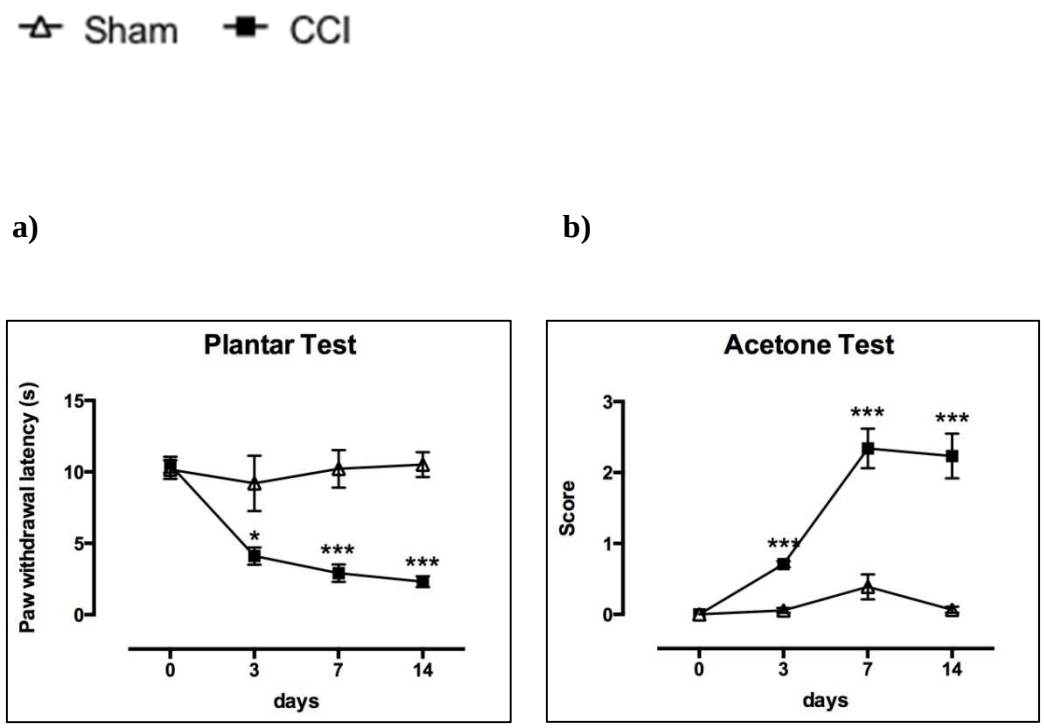


Figure 30 Determination of behavioural signs of thermal hyperalgesia a) and cold allodynia b).

a) Paw withdrawal latency recorded 0, 3, 7 and 14 days after surgery. All values are expressed as means ± standard error (n= 6 per group). Differences among groups were estimated by *t*-test. **P* < 0.05 vs Sham group.

b) Allodynic Response Score recorded 0, 3, 7 and 14 days after surgery. All values are expressed as means ± standard error (n= 6 per group). Differences among groups were estimated by Mann-Whitney U-test. **P* < 0.05 vs Sham group.

4.1.2 Gene expression studies

In the SC, gene expression studies revealed a significant increase in the mRNA levels of pDYN (1.427 ± 0.138 versus sham group equal to 1, dunnet test: $*P < 0.05$) and pBDNF (1.767 ± 0.261 versus sham group equal to 1, dunnet test: $*P < 0.05$) in the group of animals sacrified 7 days after CCI returning to values comparable with sham in the group sacrified 14 days after CCI. No significantly differences in the mRNA levels of the others genes investigated. (Figure 31a)

a) SC

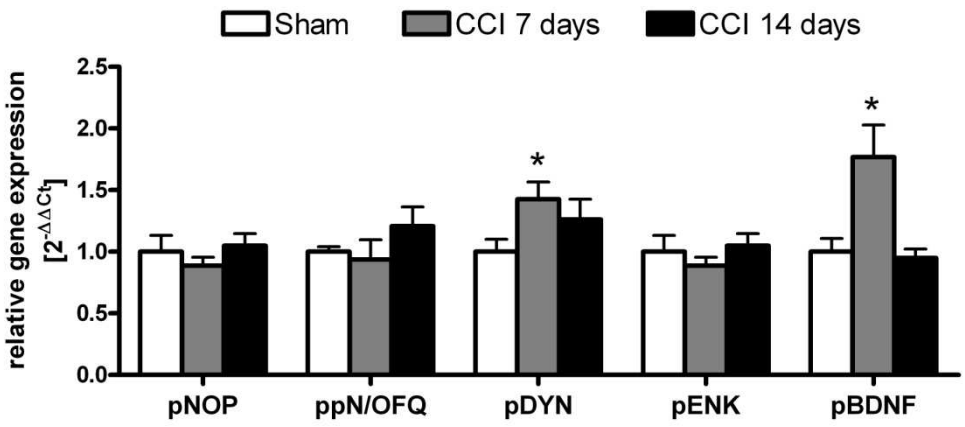


Figure 31a Levels of pNOP, ppN/OFQ, pDYN, pENK and pBDNR mRNA in the SC of CCI mice, sacrified 7 or 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test. $*P < 0.05$ versus Sham group.

In the DRGs, mRNA levels of ppN/OFQ were increased in both groups of animals sacrified 7 days and 14 days after CCI (respectively, 2.22 ± 0.52 versus sham group equal to 1, dunnet test: $*P < 0.05$; 3.59 ± 0.61 versus sham group equal to 1, dunnet test: $**P < 0.01$). Levels of the pNOP mRNA transcript was decrease in the groups of animals sacrified 7 days after CCI (0.63 ± 0.06 versus sham group equal to 1, dunnet test: $**P < 0.01$) returning to values comparable with sham in the group sacrified 14 days after surgery, whereas no changes were evident in the other genes. Levels of pDYN mRNA was not detectable (Figure 31b).

b) DRGs

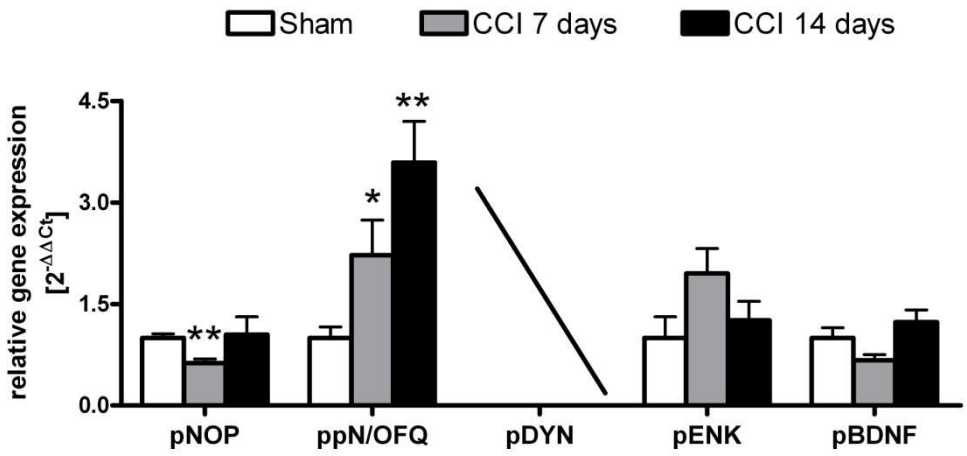


Figure 31b Levels of pNOP, ppN/OFQ, pDYN, pENK and pBDNR mRNA in the DRGs of CCI mice, sacrified 7 or 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test. $*P < 0.05$ versus Sham group.

4.2 Study of BDNF and DYN-KOPr system

4.2.1 Neuropathic BDNF transgenic mice studies

4.2.1.1 Behavioral studies

Behavioral signs of neuropathic pain a) hyperalgesia and b) allodynia were assessed by respectively Hargreaves's Plantar test and the Cold Acetone or Cold Plate Tests

a) Hyperalgesia

A similar development of hyperalgesia was observed in ICR/CD-1 and BDNF +/+ mice compared with own Sham control group, with statistically significant values at three (respectively, 3.83 ± 0.23 vs sham 9.00 ± 0.37, 3.81 ± 0.42 vs sham 7.41 ± 0.88, dunnet test $**P < 0.01$) and seven (respectively, 4.47 ± 0.27 vs sham 9.00 ± 0.37, 3.91 ± 0.31 vs sham 7.41 ± 0.88, dunnet test $**P < 0.01$) days after surgery. In contrast, a later onset of hyperalgesia was observed in BDNF +/- mice compared with Sham animals, with statistically significant values only at seven days after surgery (3.53 ± 0.37 vs sham 8.78 ± 1.09, dunnet test $**P < 0.01$) **Fig. 32**

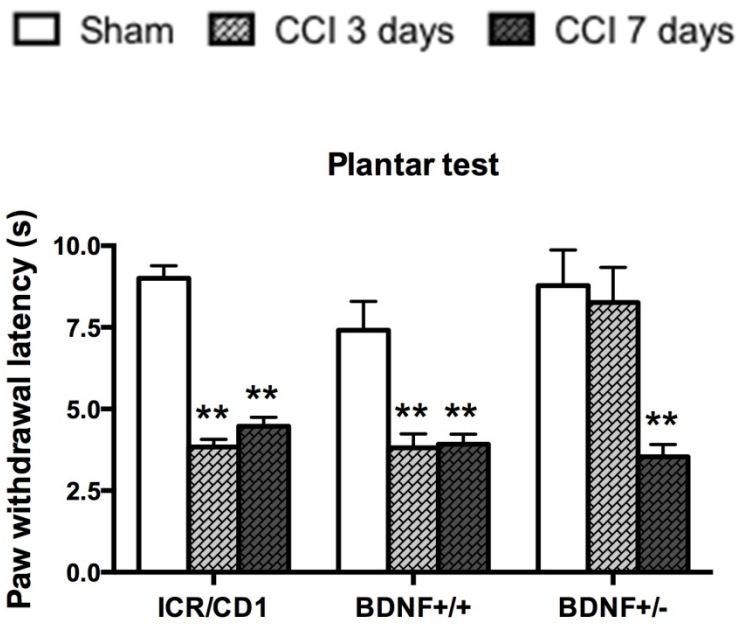


Figure 32 Determination of behavioural signs of thermal hyperalgesia.

Paw withdrawal latency recorded 3 and 7 days after surgery. All values are expressed as means ± standard error (n= 6 per group). Differences among groups were estimated by dunnet test. $^{**}P < 0.01$ versus Sham group.

b) Allodynia

BDNF +/- animals exhibited significantly lower signs of allodynia than BDNF+/+ and ICR/CD-1, each group compared with own Sham control mice, at seven days after CCI in both the cold acetone (i.e. lower scores Fig. 33a; respectively, 1.01 ± 0.35 vs Sham 0.10 ± 0.05, 1.90 ± 0.31 vs Sham 0.25 ± 0.08, 2.03 ± 0.38 vs Sham 0.38 ± 0.17, Mann-Whitney U test *P <0.05 ***P < 0.001 #P < 0.05) and cold plate the tests (i.e. higher times Fig. 33b; respectively, 13.28 ± 3.05 vs Sham 28.8 ± 0.80, 5.65 ± 0.69 vs Sham 29.2 ± 0.49, one-way ANOVA and Newman-Keuls ***P < 0.001 ##P < 0.01).

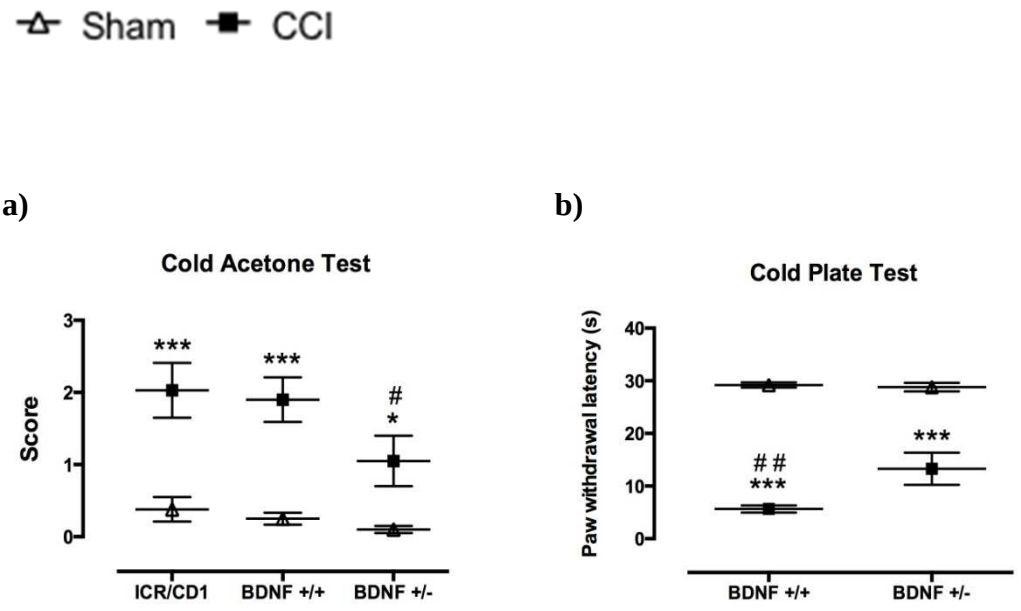


Figure 33 Determination of behavioural signs of cold allodynia.

a) Allodynic Response Score recorded 7 days after surgery. All values are expressed as means ± standard error (n= 6 per group). Differences among groups were estimated by Mann-Whitney U test. *P < 0.05 vs BDNF +/- Sham; ***P < 0.001 vs BDNF +/+ or ICR/CD-1 Sham; #P < 0.05 vs BDNF +/+ CCI 7 days.

b) Paw withdrawal latency recorded 7days after surgery. All values are expressed as means ± standard error (n= 6 per group). Differences among groups were estimated by ANOVA and Newman-Keuls. ***P < 0.001 vs BDNF +/+ or BDNF +/- Sham; ## P < 0.01 vs BDNF +/- CCI 7 days.

4.2.1.2 Gene expression

the increase in pDYN gene expression observed in the SC of ICR CD-1 mice at seven days after CCI (see Fig. 31a) was not detected in lesioned BDNF +/- mice compared with Sham animals, at the same interval Fig. 34.

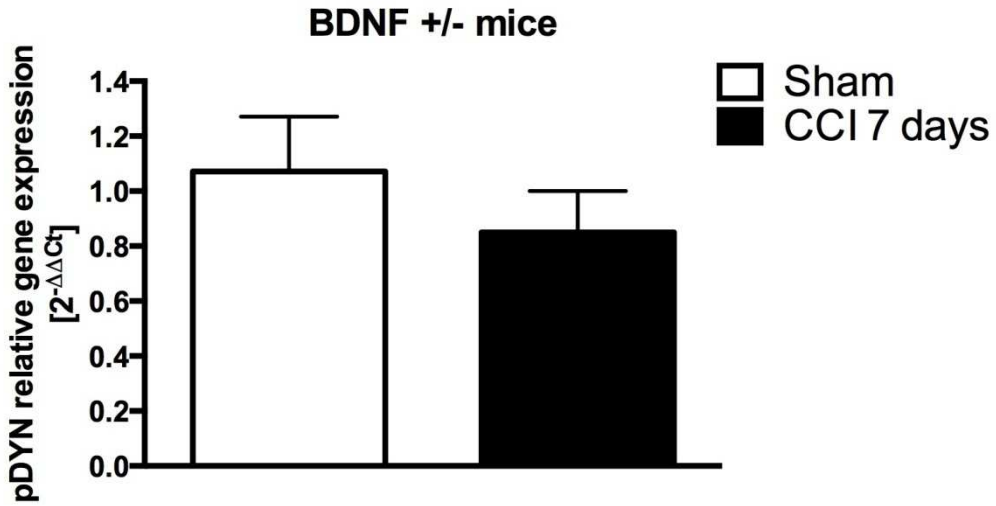


Figure 34 Levels of pDYN mRNA in the SC of BDNF +/- CCI mice, sacrificed 7 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test.

4.2.2 ICR/CD1 neuropathic mice studies

4.2.2.1 Gene expression studies

In the brain areas, gene expression studies revealed a significant increase in the mRNA levels of pBDNF in the Amy (1.74 ± 0.15 versus sham group equal to 1, unpaired t test: *P < 0.05), the ACCX (1.55 ± 0.10 versus sham group equal to 1, unpaired t test: **P < 0.01) and the PFCx (1.40 ± 0.07 versus sham group equal to 1, unpaired t test: *P < 0.05) in the CCI animals, whereas mRNA levels were significantly decreased in Th (0.51 ± 0.09 versus sham group equal to 1, unpaired t test: **P < 0.01). No changes were evident in the other areas. (Fig. 35a)

a) pBDNF

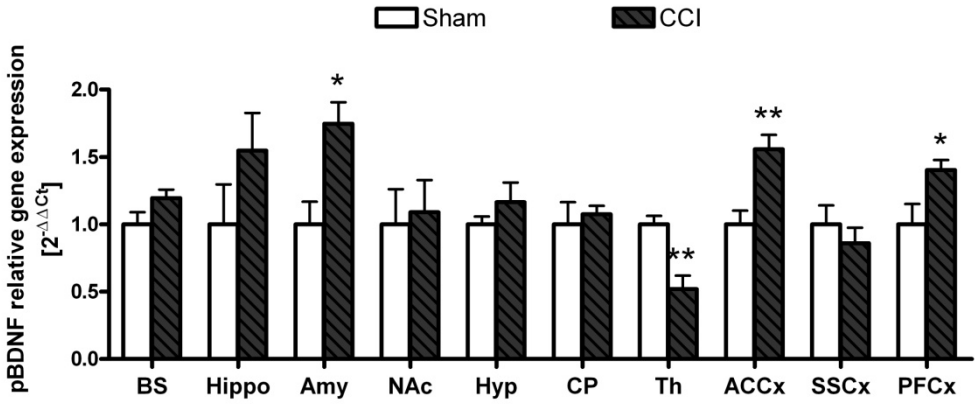


Figure 35a Levels of pBDNF mRNA in the brain areas of CCI mice, sacrified 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test. *$P < 0.05$ versus Sham group.

Levels of the pDYN mRNA transcript were increased in the CCI groups of animals in the ACCx (3.98 ± 0.39 versus sham group equal to 1, unpaired t test: ***p < 0.001) and PFCx (2.14 ± 0.021 versus sham group equal to 1, unpaired t test: *p < 0.05) whereas were decreased in BS (0.71 ± 0.05 versus sham group equal to 1, unpaired t test: *p < 0.05). No significant changes were observed in the other brain regions. (Fig. 35b)

b) pDYN

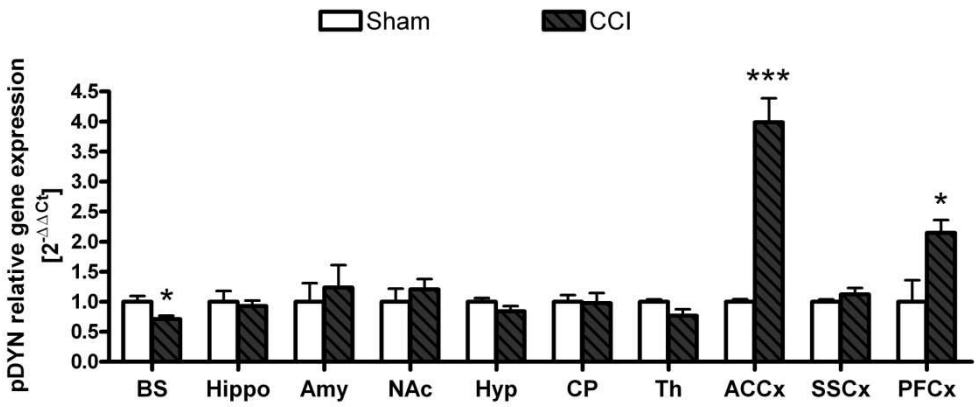


Figure 35b Levels of pDYN mRNA in the brain areas of CCI mice, sacrified 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among treatments were groups by *t*-test. *$P < 0.05$ versus Sham group.

mRNA levels of pKOP were significant decreased in NAc (0.34 ± 0.10 versus sham group equal to 1, unpaired t test: ***p < 0.001), Th (0.75 ± 0.08 versus sham group equal to 1, unpaired t test: *p < 0.05), ACCx (0.44 ± 0.10 versus sham group equal to 1, unpaired t test: *p < 0.05) and SSCx (0.50 ± 0.08 versus sham group equal to 1, unpaired t test: *p < 0.05) whereas no evident changes occurred in the other cerebral areas. (Fig. 35c)

c) pKOP

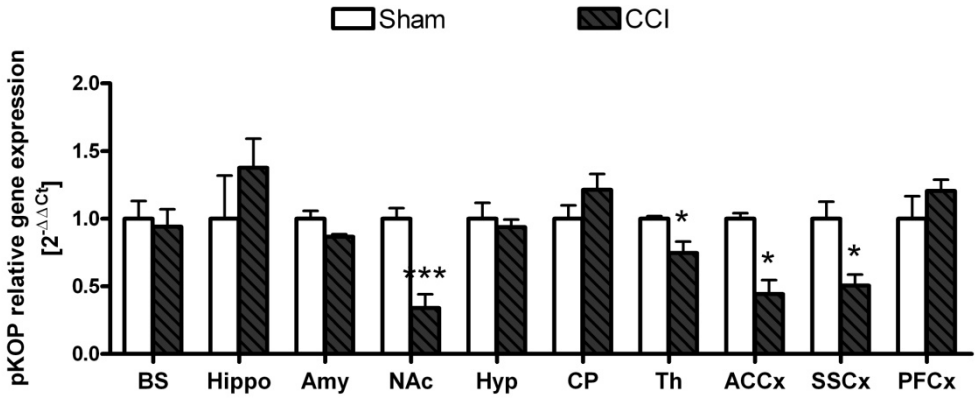


Figure 35c Levels of pKOP mRNA in the brain areas of CCI mice, sacrified 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test. *P < 0.05 versus Sham group.

4.2.2.2 Epigenetic studies: Histone modifications

Adult male ICR/CD1 mice were subjected to a right sciatic nerve lesion to investigate whether chronic pain condition could evoke epigenetic changes (histone modifications) in the pDYN and pBDNF promoter regions associated with the different previously demonstrated genes up-regulation in the SC of animals sacrified 7 days after surgery.

We found a significant decrease of H3K27me3 (0,45 ± 0,09 versus Sham = 1, p< 0,05) and H3K9me2 (0,45 ± 0,09 versus Sham = 1, p< 0,05) in pDYN promoter region as well as a significant decrease in H3K4me3 for pDYN promoter (1,7 ± 0,3, p < 0,05). No significantly alterations were observed in pBDNF promoter region (Fig. 36).

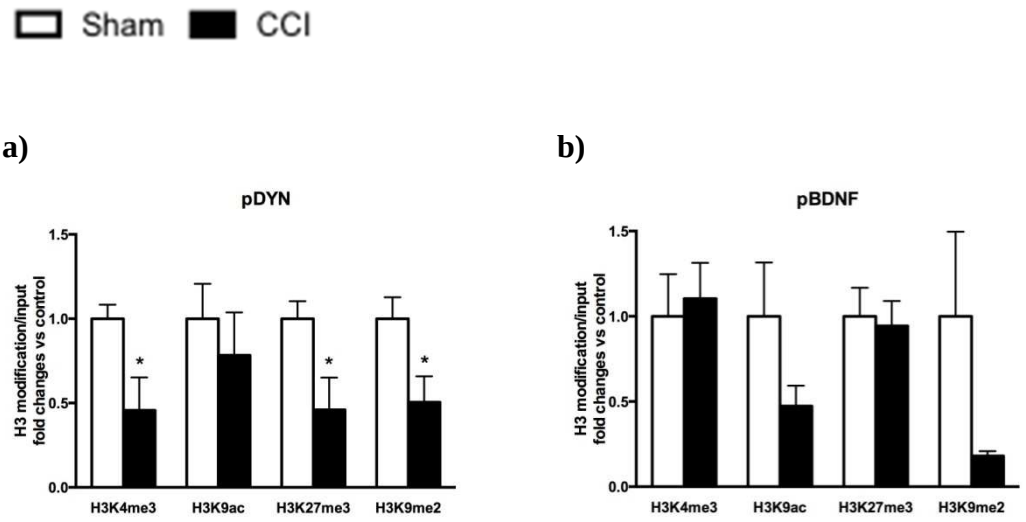


Figure 36 Real Time-qPCR analyses of H3K4me3, H3K9Ac, H3K27me3 and H3K9me2 immunoprecipitated DNA fragments at a) pDYN and b) pBDNF promoters. ChIP showing the levels of specific histone modification normalized to total input DNA in CCI mice (n =6) or Sham mice (n =6). Data are expressed as means ± SE of triplicate independent samples.*P < 0.05 vs. Sham; t-test.

4.3 Study of N/OFQ-NOPr system

4.3.1 Gene Expression

In cerebral areas, levels of the ppN/OFQ mRNA transcript were segnificantly decreased in BS (0.65 ± 0.02 versus sham group equal to 1, unpaired t test: *p < 0.05), Hippo (0.76 ± 0.05 versus sham group equal to 1, unpaired t test: *p < 0.05), Amy (0.71 ± 0.07 versus sham group equal to 1, unpaired t test: *p < 0.05), Nac (0.39 ± 0.08 versus sham group equal to 1, unpaired t test: *p < 0.05) and CP (0.38 ± 0.05 versus sham group equal to 1, unpaired t test: *p < 0.05. No significantly changes were observed in others brains areas (Fig 37a).

a) ppN/OFQ

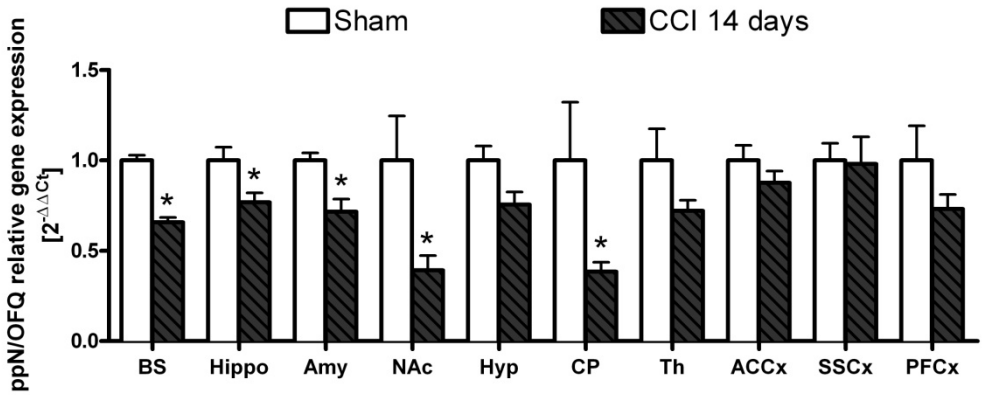


Figure 37a Levels of ppN/OFQ mRNA in the brain areas of CCI mice, sacrified 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test. *$P < 0.05$ versus Sham group.

pNOP gene expression was segnificantly decreased in BS (0.54 ± 0.08 versus sham group equal to 1, unpaired t test: *p < 0.05), and CP (0.57 ± 0.12 versus sham group equal to 1, unpaired t test: *p < 0.05. No significantly changes were observed in others brains areas (Fig. 37b).

b) pNOP

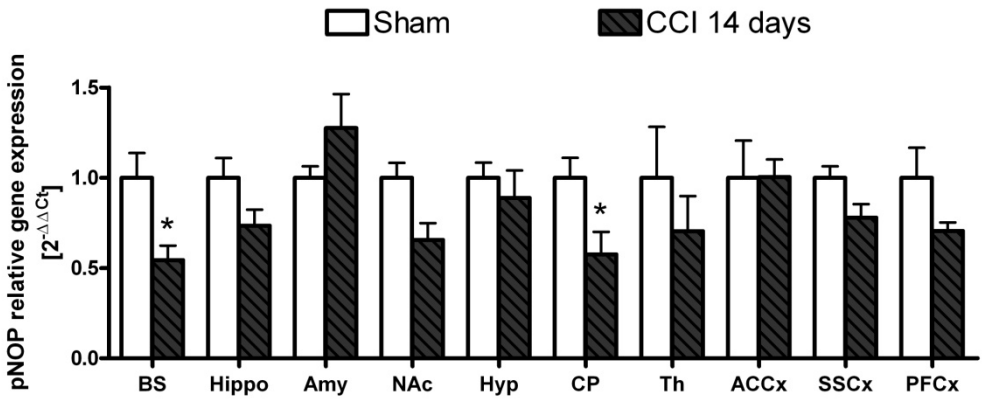


Figure 37b Levels of pNOP mRNA in the brain areas of CCI mice, sacrified 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method of six mice for each group. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and means of mRNA levels are expressed relative to Sham mice ± standard error of the mean. Differences among groups were estimated by *t*-test. *$P < 0.05$ versus Sham group.

4.3.2 Quantification of active peptide levels

In Amy, levels of ir-N/OFQ were significantly higher in CCI group compared with Sham animals (15153.74 ± 2215.96 versus 7504.56 ± 284.85, unpaired t test: *p < 0.05) whereas no significant alteration were observed in other brain regions (Fig. 38).

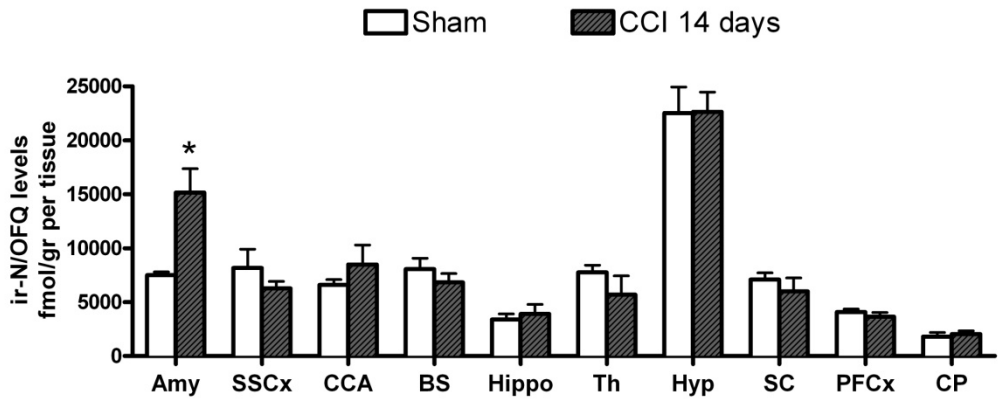


Figure 38 N/OFQ levels in brain areas of CCI mice, sacrified 14 days after surgery, in comparison with Sham groups, see Materials and Methods section for details. All values are expressed as means ± standard error (n= 5 per group). Differences among groups were estimated by t-test. *P < 0.05 versus Sham group.

4.3.3 NOP-EGFP mice: charaterization and validation of knock-in mouse strain

Knock-in mice were constructed so that the NOP receptor with eGFP fused at the C-terminus is expressed in place of the native receptor with eGFP attached to the C-terminal of NOP receptors.

4.3.3.1 Primary Cultures: Internalization studies

Internalization studies conducted on hippocampal or cortical primary neurons showed that 1.0 μM N/OFQ addition causes internalization of receptors.
Fig. 39 (top view of the neuron cell) shows that fluorescent signal from NOP-eGFP gradually disappear at 0, 5 min and 15 min after drug addition, from the cell membrane to the inside of the cell.

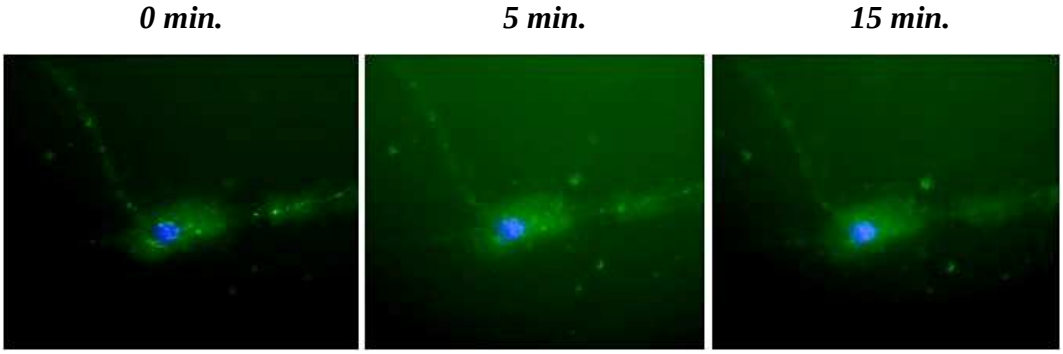


Figure 39 Real-time Fluorecense Imaging of N/OFQ (μM)-induced NOP-eEGFP redistribution in primary cortical neurons. A representative experiment is shown (n= 4 referring to cell cultures from pools of 4 to 6 mouse pups). Images at 0, 5 and 15 min of treatment (Magnificence 60x).

Similarly, cells showed in Fig. 40 and Fig. 41 (observation of the equatorial plane of the neuron cells) displayed a rearrangement of the NOP-eGFP signal, from diffused around cell body and processes to dots organized , from 0 to 20 minutes after drug addition.

0 min. ***20 min.***

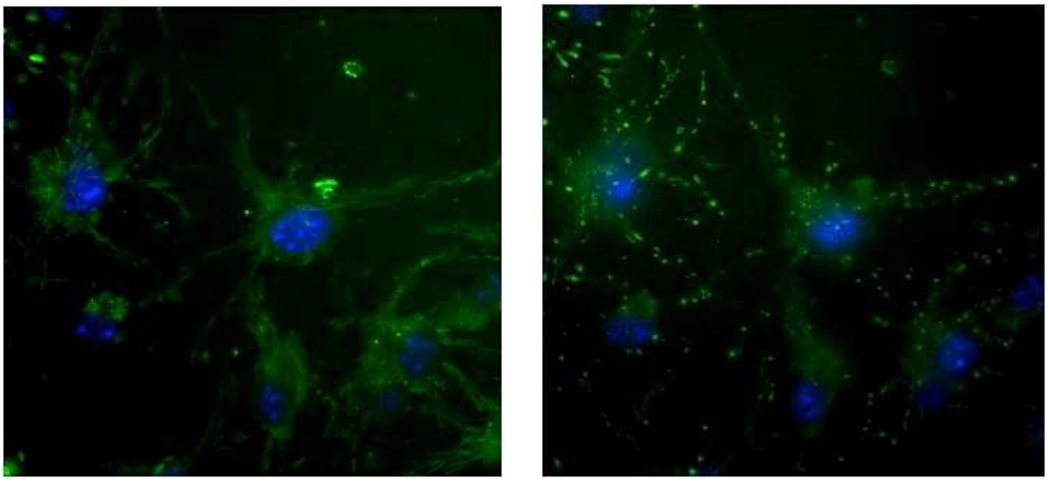


Figure 40 Real-time Fluorecense Imaging of N/OFQ (1µM)-induced NOP-eEGFP redistribution in primary hippocampal neurons. A representative experiment is shown (n= 4 referring to cell cultures from pools of 4 to 6 mouse pups). Images at 0 and 20 min of treatment (Magnificence 60x).

0 min. ***20 min.***

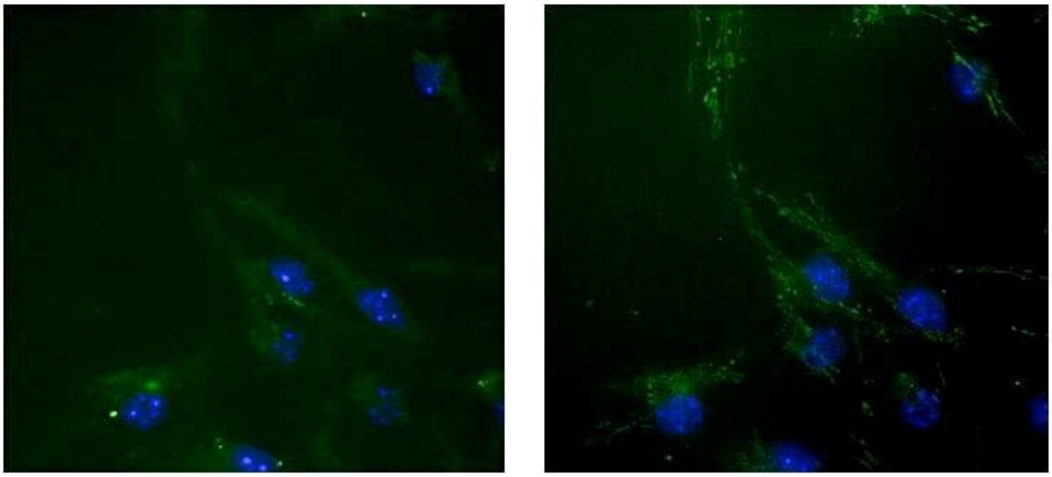


Figure 41 Real-time Fluorecense Imaging of N/OFQ (1µM)-induced NOP-eEGFP redistribution in primary hippocampal neurons. A representative experiment is shown (n= 4 referring to cell cultures from pools of 4 to 6 mouse pups). Images at 0 and 20 min of treatment (Magnificence 60x).

Cell Neuron in Fig. 42 shows a high signal from NOP-EGFP already internalized at time 0 before drug addition.

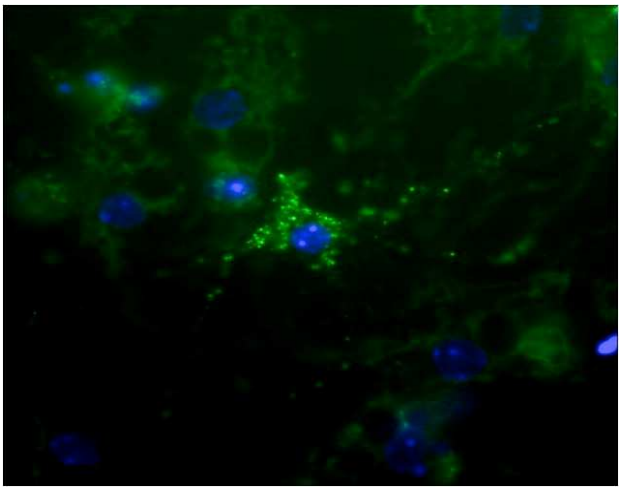


Figure 42 Real-time Fluorecense Imaging of primary cortical neurons.

4.3.3.2 Brain Sections: Localization studies

Analysis of brain slices showed that the receptors appear to be located in brain regions, such as a) cingulate cortex (Cg) and b) hippocampus (Hippo), previously demonstrated to contain NOP receptor mRNA and NOP receptor binding sites.

a) Cg, in Fig. 43a is shown Cg2 (left top) to the edge with the white matter (right bottom). Fluorescent signal was present in in neuron of the Cg2 but not cell of the white matter. In Fig 43b is shown Cg1 region. Fluorescent signal was present around neurons and in connected cell processes.

a) *b)*

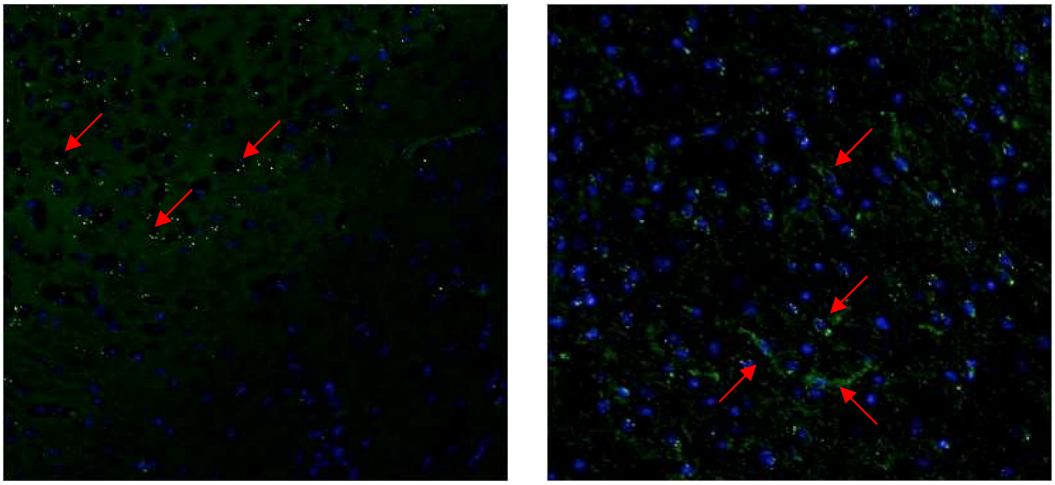


Figure 43 Anatomical distribution of fluorescence in NOP-eGFP mice.

a) Fluorecense Images of Cg2 at the edge with white matter.

b) Fluorencense Imges of Cg1 region.

b) Hippo, in Fig 44a, b, c is shown NOP-eGFP signal around neuron cell body and connected cell processes, in different hippocampus areas.

a) *b)* *c)*

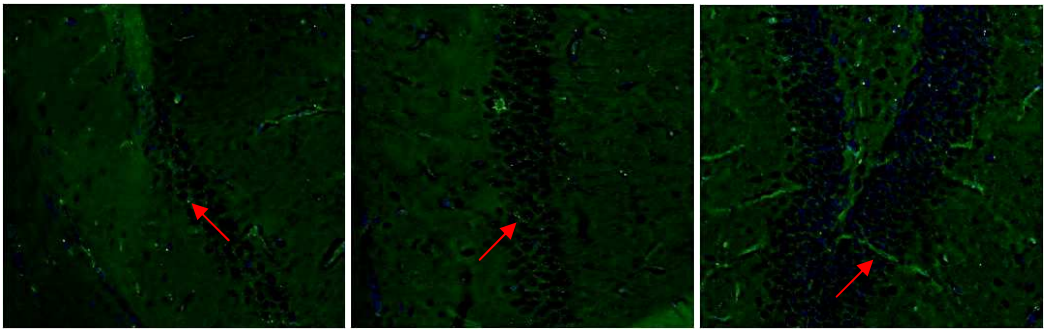


Figure 44 Anatomical distribution of fluorescence in NOP-eGFP mice. Fluorecense Images of Hippo.

5. DISCUSSION

Neuropathic pain has been described as the "*most terrible of all tortures which a nerve wound may inflict*" and arises as a consequence of nerve injury either of the peripheral or central nervous system. Following peripheral nerve injury, a cascade of events in the primary afferents leads to peripheral sensitization resulting in sensory abnormalities including stimulus-independent persistent pain or abnormal sensory perception such as hyperalgesia and allodynia *(Woolf C.J., Mannion R.J. 1999).*

The precise mechanisms underlying neuropathic pain and the relationship among different mechanisms are not fully understood. Peripheral nerve injury is followed by a change in expression of neurotransmitters, neuromodulators, growth factors and neuroactive molecules in primary afferent neurons located in dorsal root ganglion of the spinal cord. These changes in-turn induce sensitization of primary afferents inputs (peripheral sensitization) leading to exaggerated pain perception in an injured tissue or territory innervated by an injured nerve. Apart from the pain hypersensitivity in an injured tissue, neuropathic pain also spreads to the adjacent non-injured extra-territory regions (extraterritorial pain) and contralateral parts (mirror image pain) *(Woolf C.J., Mannion R.J. 1999)* indicating the esistence of maladaptive changes in neural network in the central nervous system (central sensitization). The induction of central sensitization has been identified at the spinal cord level (dorsal horn neurons, second order neurons) and in the brain regions (third order neurons). A number of reports have suggested the neuroplastic changes in different brain areas that are responsible for central sensitization and behavioral alterations in peripheral nerve injury-induced neuropathic pain *(Jaggi A.S., Singh N. 2011).*

Development of animal models has contributed immensely in understanding the chronic pain and underlying peripheral as well as central pathogenic mechanisms. The ideal animal models should result in reproducible sensory deficits such as allodynia, hyperalgesia and over a sustained period. In the present study we used chronic constriction injury to the sciatic nerve (CCI), which more closely resembles behavior (hyperalgesia and allodynia) of peripheral neuropathy in rodents (*Colleoni*

M., Sacerdote P. 2010). The first result of the present study is that the experimental conditions used were able to evoke neuropathic signs of hyperalgesia and allodynia for a prolonged period (i.e. 14 days). Therefore our results proved that we were in the right experimental conditions.

Although the presence of many evidences as described above *(Jaggi A.S., Singh N. 2011),* it is generally difficult to clearly understand whether changes in RNA levels reflect a possible involvement of a specific gene in chronic pain neuroplasticity. The main goal of our study was to try to correlate the effects of peripheral nerve injury on opioids and BDNF gene expressions.

BDNF and DYN-KOPr system

Many studies examined dynorphinergic system and BDNF activity during neuropathic pain conditions *(Mika J. et al. 2011; Vanelderen P. et al. 2010).*

Although dynorphin is commonly characterized as an endogenous opioid peptide, considerable evidence reveals that dynorphin is actually pronociceptive in chronic pain states *(Wang Z. et al. 2001).* Several lines of evidence proposed a central role for BDNF in the initiation of central sensitization caused by an alteration of the Cl^- cell gradient followed by the inversion of GABAergic inhibitory activity *(Kohno T. et al. 2005)* and by an increase in the NMDA receptors-mediated excitatory activity suggesting a pro-nociceptive effect of this neutrophin *(Vanelderen P. et al. 2010).* Here we observed a transient up-regulation of both pDYN and pBDNF mRNA levels in the SC of injured animals at seven days after surgery returning to values comparable with control animals at fourteen days. pDYN gene expression data appear to be consistent with the hypothesis that elevated spinal dynorphin is required for maintaining neuropathic pain suggesting that endogenous dynorphin is a key mediator of some aspects of neuropathic pain states *(Lai J. et al. 2001).* pBDNF data seem to be related to the possibility previously proposed that BDNF functions as a final common path for a convergence of perturbations that culminate in the generation of neuropathic pain. Peripheral nerve injury activates spinal microglia. Although a variety of neuropeptides, cytokines, chemokines and neurotransmitters have been implicated at various points in this process, much of the information

transfer between activated microglia and neurons, at least in this context, may be explicated by BDNF *(Biggs J.E. et al. 2010)*. Moreover, the simultaneous up-regulation of pDYN and pBDNF seems sustain the hypothesis recently proposed that dynorphin could act as a downstream effector of some BDNF actions *(Logrip M.L. et al. 2008).*

These data indicate that some modifications of the biosynthesis of neurotransmitters highly involved in this pathology occur early after the injury and cannot be more evident at later intervals.

In parallel, behavioural and neurochemical data obtained in BDNF transgenic mice seem confirmed the hypothesis proposed above. A similar development of hyperalgesia (plantar test) was observed in ICR/CD-1 and BDNF +/+ (wild type) mice. In contrast, a later onset of hyperalgesia was observed in BDNF +/- mice (BDNF lacking mice). In addition, BDNF +/- animals exhibited significantly lower signs of allodynia than BDNF +/+, in both the cold plate (i.e. higher times) and the acetone tests (i.e. lower scores). These behavioral results support a role for BDNF in mechanisms underlying the development of allodynia and hyperalgesia, thus confirming its involvement in neuroplasticity leading to neuropathic pain conditions. Furthermore, the increase in pDYN gene expression observed in the spinal cord of ICR/CD-1 mice at seven days after CCI was not detected in lesioned BDNF +/- mice, at the same interval. This result suggest some relationship between BDNF and DYN and seems to confirm the existence of a possible BDNF and DYN cross-talk.

Since epigenetic mechanisms seem to play a role in neurol plasticity resulting from chronic pain *(Chwang W.B. et al. 2007; Koshibu K. et al. 2009; Lubin F.D. et al. 2008),* we here also studied the involvement of epigenetic mechanisms in the pDYN and pBDNF genes expression changes observed seven days after surgery in the SC. We observed a close relationship between selective chromatin modifications and pDYN genes expression. In particolar, our study revealed a decrease of H3K27me3 and H3K9me2, repressive marks, in the pDYN gene promoter region. These data are in agreement with the gene expression increase previously observed in the same interval after the nerve injury. However, concerning activating marks, we observed a decrease of H3K4me3 in the pDYN gene promoter region and no changes of H3K9ac. Moreover, no changes of histone modifications were observed in the

pBDNF gene promoter region. These last evidences seem to be not directly associated with gene expression changes. This could be possibly explained by different hypothesis. First of all, it has to be taken under consideration the complexity of gene regulation by histone modifications *(Barski A. et al. 2007)* and that multiple modifications may function cooperatively to prepare chromatin for transcriptional activation.

One possibility for both pDYN and pBDNF gene could be found in the already mentioned and underlined transient nature of their mRNA increase. Could be possible that at the time of investigation activating phenomena are in a shutdown process, while repressive phenomena are still inactive. Moreover, the already mentioned role of downstream effector of DYN in BDNF acting, could explain why epigenetic mechanisms are still operating in pDYN gene promoter region, while no changes of histone modifications are present in pBNDF gene promoter region.

The peripheral nerve injury has been also reported to induce neuroplastic changes in different brain regions *(Jaggi A.S., Singh N. 2011)*. Here we also investigated gene expression alterations in different brain regions, fourteen days after surgery when the painful condition is fully established. Results indicated that evident alterations of the investigated systems occur. In particular, pDYN gene expression was found to be up-regulated in the ACCx and PFCx of the CCI mice, while his mRNA levels were found to be significantly lower than in control animals in the BS. At the same interval, pKOP receptor biosynthesis was found to be down-regulated in the NAc, the Th, the ACCx an SSCx. These data seem to further confirm the involvement of DYN-KOPr system in neuroplasticity occurring in neuropathic pain. Alterations in the ACCx appear particularly interesting. The higher pDYN up-regulation and the simultaneous down-regulation of the pKOP receptor suggest that a pronounced increase in the ligand availability lead to a down-regulation of his receptor.

The decrease of pDYN level in the BS could be related with the alterations of functionality of the descending control systems of nociception for which important and fine regulation mechanisms exist at this neuraxis level.

Concerning BDNF alterations, particulary interesting is the simultaneously up-regulation of both pBDNF and pDYN mRNA levels in the same brain areas (ACCx

and PFCx). These data could further confirm the close relationship between the these two neuropeptidergic systems.

Experiencing stressful life events, such as chronic pain, increase the risk of later developing anxiety and depressive disorders. While it is well established that Amy, PFCx and Hippo are key brain structures in fear memory processing *(McGaugh J.L. 2004; Herry C. et al. 2008; Likhtik E. et al. 2008),* it is not well known which neurotransmitters or neuromodulators are involved. In the present study we observed increased pDYN mRNA in the PFCx of injured mice that seems to be consistent with a recently proposed role for the dynorphin in the extinction of negative fear memories related to stressful situations *(Bilkei-Gorzo A. et al. 2012)*, such as neuropathic pain. Once again, we also observed pBDNF up-regulation in the Amy of CCI animals in agreement with a recent study proposing BDNF as a mediator of susceptibility of fear conditioning in the Amy.

N/OFQ-NOPr system

Nociceptine system is widely recognized to be involved in chronic pain *(Mika J. et al. 2011).* Despite the many advances in our understanding of the involvement of different neuropeptide systems in pain, the precise function of this system has not yet been fully characterized. It has been clearly documented that the induction of chronic pain states, especially neuropathy, is associated with an up-regulation of N/OFQ, as indicated by enhanced expression of p/NOFQ protein and/or mRNA *(Mika J. et al. 2010).* Moreover, it has been shown that neuropathic and other chronic pain states are associated with elevations of DRG content for N/OFQ *(Mika J. et al. 2010).* Consistent with these evidences, here we observed an up-regulation of ppN/OFQ in the DRGs of injured mice seven days after surgery, with further increased values at fourteen days. In contrast, we also found a down-regulation of pNOP in DRGs of CCI mice seven days after surjery, returning to values comparable with sham at fourteen days. This result could be explained as an enhanced increase in the ligand availability lead to a down-regulation of his receptor.

As already mentioned, a damage to a peripheral nerve is able to induce neuroplastic changes in different brain regions *(Jaggi A.S., Singh N. 2011).* For this reason we

also investigated gene expression alterations in different brain regions, fourteen days after surjery that is at the time when we observed a pronounced ppN/OFQ mRNA up-regulation in the DRGs.

The decreased of both ppN/OFQ and pNOP mRNAs levels observed in the BS could be related to nociception. Indeed, although this area is involved in the modulation of wide range of brain functions, the modulation of descending control system of nociception is one of the most important in this context. In this area, nociceptive transmission is directly controlled by the gigantocellular paragigantocellular nuclei as well as by the raphe magnus nucleus.

Concerning the other brain regions investigated, gene expression changes seem to be more relevant in brain areas associated with the modulation of the affective-emotional component of pain than with areas related to the modulation of nociceptive transmission. In particular, we observed down-regulation of ppN/OFQ mRNA levels in the Hippo, Amy, NAc and CP. Furthermore, we also observed a decrease of pNOP gene expression in the CP.

As already mentioned neuropathic pain cause neuroplastic changes in several brain areas including the amygdala and the striatum *(Jaggi A.S., Singh N. 2011).* Indeed, neuroplastic changes in the amygdala have been suggested to be responsible for the development of depressive-like behavior in subjects affected by prolonged painful conditions *(Gonçalves L. et al. 2008)*. Moreover, morpho-functional alterations have recently been described in the Hippo, together with anxiety behavior changes, in a mouse model of neuropathic pain *(Mutzo A.A. et al. 2012)*. ppN/OFQ gene expression alterations observed in these areas seem to be consistent with the already known involvement of this system in the anxiety/depressive states, proved by the anxiolytic and antidepressant effects respectively exerted by NOP agonists and antagonists *(Calò G. et al. 2000)* as well as by the reduced anxiety-like behavior in restraint rats after N/OFQ microinjection into the central nucleus of Amy *(Ciccocioppo R. et al. 2014)*.

In addition, concerning changes observed in the NAc and CP, it is interesting to note that these areas include neuronal pathways modulate through dopamine important central functions such as reward/aversion (in particular in the NA), but also descending control system of nociception (CP) *(Jaggi A.S., Singh N. 2011).* The

involvement of the N/OFQ-NOPr system in the mechanisms of reward/aversion *(Xu X. et al. 2000),* together with the evidence that N/OFQ is able to attenuate both the basal and the cocaine-induced increase in extracellular DA in the NAc *(Lutfy K. et al. 2001)* and the certainly aversive condition represented by neuropathic pain could explain, at least in part, the involvement of this system in NAc. On the other hand, changes observed in the CP could be explained with the well-known involvement of the investigated system in the control of nociception. In this context, alteration in the levels of the inhibitory neuro-mediator N/OFQ could easily affect the release of mediators such as GABA or excitatory amino acids that play a crucial role in the functionality of the descending system.

In addition, here we also studied alterations of bioactive N/OFQ levels in the same brain regions, in the same experimental conditions. We were able to observe elevated N/OFQ levels in the Amy of injured mice compared to Sham animals, whereas no significant peptide levels changes were observed in the other investigated brain areas. This result seems to be related with the proposed involvement of both Amy and Nociceptin system in the development of depressive-like behavior in subjects with chronic pain. On the other hand, the elevated bioactive N/OFQ levels seem to be in contrast with the its simultaneously gene expression down-regulation. This inconsistency could be explained in term of negative feed-back since pronounced increase in the bioactive peptide availability lead to a down-regulation of its precursor.

NOP-EGFP mice

Internalization of NOP receptors has been studied in transfected cells, using various fusion proteins, including C-terminal fused GFP. In transfected cells NOP receptor agonists induce internalization *(Spampinato S. et al. 2002).* Moreover, NOP receptors apparently can heterodimerize with all of the opiate receptors. NOP receptor activation was found to induce internalization of μ, δ, and κ receptors when co-transfected into HEK cells *(Evans R.M. et al. 2010).* The formation of opioid/NOP receptor heterodimers could have profound effect on nociceptive processing. However, studying these phenomena in a native system has been

impossible. Kieffer and colleagues produced the first transgenic mice containing the delta opioid receptor with enhanced Green Fluorescent Protein attached on the C-terminal *(Scherrer G. et al. 2006)*. The visualization of receptors has long been a central part of the understanding of a receptor system. Visualization can provide information as to the location and trafficking of these important membrane proteins, leading to a better understanding of the activation and inactivation of the receptors. Here we focused our attention on the characterization and validation of the NOP-EGFP mice strain. This study in currently in progress and here we presented preliminary data. We observed that N/OFQ somministration was able to induce NOP receptor internalization in primary neurons. However, we also found NOP receptor already internalized in different cells before drug addition, suggesting that in basal conditions receptor could be organized in storage vesicles. Analysis on brain slices showed that the receptors appear to be located in brain regions, previously demonstrated to contain NOP receptor mRNA and NOP receptor binding sites *(Neal Jr C.R. et al 1999)*. In particular, we observed that EGFP signal was specific in the Cingulate cortex and in the Hippocampus. Moreover, specific fluorescent signal was present around neuron cells and in cell connecting processes.

6. CONCLUSIONS AND PERSPECTIVES

Chronic pain is one of the most prevalent health problems in our modern world. Affecting approximately one in five adults, chronic pain is associated with a significantly reduced quality of life and a higher risk of depression and other mental health disorders *(Breivik H. et al. 2006; Gureje O. 1998).*

It has not been easy to develop novel and effective classes of analgesic drugs - there have been almost no new registrations in the past 15 years. There has been much discussion about the reasons for past failures and this has stimulated an interest in exploring novel mechanisms. There are three main biological mechanisms that contribute to persistent pain: peripheral sensitization of primary nociceptors within the dorsal root ganglion; central sensitization of spinal interneurons; and descending modulation of the pain signal from the brainstem and higher cortical centers. At all levels of processing, significant cellular and molecular changes occur, such as large alterations in the transcriptional profile of these tissues *(Crow M. et al. 2013).*

The data on BDNF and DYN-KOPr system confirm a role of these system in neuronal plasticity resulting from chronic pain *(Biggs J.E. et al. 2010; Lai J. et al. 2001)* and the hypothesis of a close relationship between the these two neuropeptidergic systems *(Logrip M.L. et al. 2008)*.

The linkage between gene expression alterations and epigenetic modulation in pDYN and pBDNF promoters following nerve injury confirm the possible involvement of chromatin remodeling mechanism in chronic pain neuroplasticity *(Denk F., McMahon S.B. 2012)*.

The data on N/OFQ-NOPr system confirm the involvemente of this system in the neuroplastic alterations underlying the development and maintenance of chronic/neuropathic pain and also suggest its involvement in the regulation of mood disorders accompanying the chronic painful state.

The characterization of the NOP-EGFP mice confirm the usefulness of this innovative transgenic mouse strain for the receptor visualization in native tissue, therefore internalization and other trafficking can be seen in real time.

Overall, our results could be important to partially fill the lack of knowledge of which and how neuropeptidergic systems are involved in neuroplastic mechanism

occurring in neuropathic pain that lead to establish the chronic condition and suggest the possibility of using drugs acting on these systems for the treatment of this invalidating desease.

A deepened knowledge of the neuroanatomy, neurophysiology, neurochemistry and neuroplasticity of chronic/neuropathic pain is needed to identify new targets allowing the development of innovative therapeutic strategies for the prevention of neuropathic pain establishment.

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