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CICLO

XXVI

Study of autophagic and epigenetic mechanisms in experimental models of inflammatory and neuropathic pain for the identification of new pharmacological targets

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A mio padre, a mia madre

"Tutto posso in Colui che mi dà la forza"

(Fil 4,13)

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Abbreviations

5-HT	5-hydroxytryptamine
5hmC	5-hydroxymethylcytosines
5mC	5-methylcytosine
ACTH	Adrenocorticotropic hormone
AD	Alzheimer Disease
AMPK	AMP-activated protein kinase
ATG	Autophagy-related genes
BCL-2	B-cell lymphoma 2
CaMKII	Calmodulin-Dependent Protein Kinase II
CCI	Chronic Constriction Injury
CFA	Complete Freud's Adjuvant
CGRP	Calcitonin gene-related peptide
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CRH	Corticotrophin-releasing hormone
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
DRG	Dorsal root ganglia
ES	Embrional stem
FKBP5	FK506 binding protein 5
GABA	Gamma-Aminobutyric acid
GAD65	Glutamic Acid Decarboxylase
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
GRE	Glucocorticoid Response Element
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
Hsp90	Heat shock protein 90
HTM	High-threshold mechanical nociceptors
IASP	INTERNATIONAL ASSOCIATION FOR THE STUDY OF PAIN

IL-6	Interleukin 6
IP3	Inositol triphosphate
JNK	Jun N-terminal kinase
LC3	Microtubule-associated protein light chain 3
LDP	Long term depression
LTP	Long term potentiation
MeCP2	Methyl-CpG binding protein 2
mGluR2	Metabotropic glutamate receptor 2.
mTOR	Mammalian target of rapamycin
N-VGCC	N-type voltage-gated calcium channel
Na_v 1.7	Voltage-gated sodium channel
NF-Kb	Nuclear factor-kappa B
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartate
NP	Neuropathic Pain
NSAID	Non-steroidal anti-inflammatory drug
PAG	Periaqueductal gray region
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E_2
PI	Phosphatidylinositol
PI3P	Phosphatidyl inositol triphosphate
PTSD	Post-Traumatic Stress Disorder
RNA	Ribonucleic acid
RVM	Rostral ventromedial medulla
SCN9A	Sodium channel protein type 9 subunit alpha
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SNI	Spared Nerve Injury
SNL	Spinal nerve Ligation
SNPs	Single-nucleotide polymorphisms
Tet1	Ten-eleven translocation 1
TNF	Tumor necrosis factor
TORC1	Multiprotein TOR complex 1
UPS	Ubiquitin proteasome system

SUMMARY

Pain is defined by IASP as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 2011). While physiological pain is like a warning system, useful to prevent damage to the organism, pathological pain is an unpleasant sensation, permanent also after damage and it is characterized by an enhanced sensitivity to both innocuous and noxious stimuli (termed allodynia and hyperalgesia, respectively). While acute pain resolves in few days, chronic pain lasts longer than three/six months**.** Neuropathic pain, a common form of chronic pain, was defined as "pain caused by a lesion or disease of the somatosensory nervous system" (IASP, 2011). Pharmacological treatments available, including tricycles antidepressant and gabapentin, have limited efficacy in most of patients (Childers et al, 2007). Therefore, a better understanding of pain physiopathology and the development of new treatments are very important. Here, we characterised two new molecular mechanisms, autophagy and epigenetic mechanisms, and their role in pain processing. Autophagy is the main mechanisms involved in the degradation of proteins and organelles, in cell remodelling and survival during periods of nutrient deficiency. The decrease in the autophagic activity seems to interfere with the degradation of proteins and with the turnover of nutrients, while a greater activation of this pathway appears to facilitate the clearance of protein aggregates and to promote neuronal survival in various neurodegenerative diseases. On the other hand, too high autophagic activity can be detrimental and lead to cell death, suggesting that the regulation of autophagy has an important role in determining cell fate. However, despite numerous studies on the role of autophagy in neurodegenerative diseases, the role of this process in the pathophysiology of neuropathic pain remains poorly studied.

Epigenetic mechanisms are chemical modifications of chromatin that influence gene expression without altering the DNA sequence. Although in recent years scientific research has produced significant results in the epigenetics field, only few studies have focused on the involvement of epigenetic mechanisms in relation to pain states. Experimental evidence suggests that changes in the expression of some genes are involved in the early stages of induction and maintenance of chronic pain states. Among these genes, recent evidence suggests a role for the FKBP5 gene, an important

regulator of the glucocorticoid receptor, involved in the regulation system of the stress response. In addition, recent studies show that this gene is under strong epigenetic control. In view of this, the objectives of this research were:

- To characterise the autophagic process at spinal cord level in different experimental models of neuropathic and inflammatory pain;
- To verify the relevance of spinal autophagy for pain processing;
- To identify pain conditions in which the gene FKBP5 plays a role;
- To study the role of FKBP5 on pain processing at spinal cord level;
- To characterize the enzymes involved in DNA methylation;

The results obtained in the first experimental part of this thesis showed a modulation of the main autophagic markers in experimental models of neuropathic pain. In particular, in the model that involves the ligation of the L5 spinal nerve (SNL) and in the model that involves the transection of the tibial nerve and peroneal (SNI), it was observed an increase in the levels of the associated form of the protein LC3 (LC3II) and of protein p62 , which is involved in the early stages of degradation of the autophagic process. The observed increase in p62 protein levels suggested a possible impairment of autophagic flux. To verify this hypothesis the consequences of a local block of autophagy at spinal level were investigated on pain behaviour. In particular, the treatment of naïve animals with chloroquine, a lisosomal inhibitor, resulted in the establishment of a state of hyperalgesia typically observed after peripheral damage of the spinal nerves. The results obtained in the second experimental part demonstrate an involvement of the gene FKBP5 in the induction and in the maintenance phases of chronic pain. In particular, knockout animals have shown a lower sensitivity to mechanical stimuli following the onset of various chronic pain states. The silencing of the gene at the spinal cord level has allowed us to understand the role of the gene FKBP5 in pain processing after an injury. Finally, the study and characterization of DNMT1, the enzyme involved in DNA methylation, has allowed us to suggest the active involvement of other proteins in the process of DNA demethylation and then in the expression of genes. In conclusion, the data reported in this study indicate an impairment of autophagy in experimental models of neuropathic pain, supporting the neuroprotective role of this process in the

spinal cord. It was also demonstrated the involvement of the gene FKBP5 in the induction and in the maintenance phases of chronic pain. Altogether, these data pave the way to further investigations aimed to a better understanding of the mechanisms underling chronic pain and to the identification of potential molecular targets for the development of new therapeutic strategies.

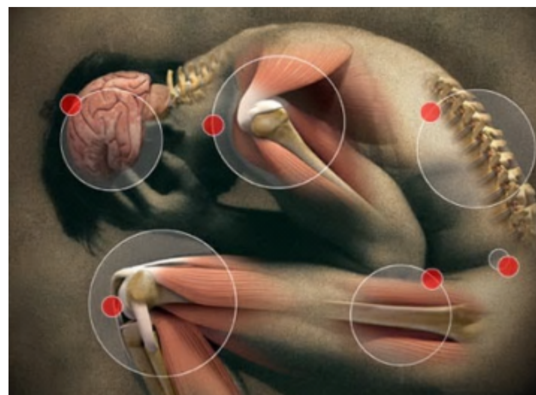
Introduction

The nature of pain has intrigued philosophers for millennia. The ancient Greeks conceived pain as an emotion. In the late nineteenth and early twentieth centuries, the view of pain as sensation became preeminent: it was seen as a direct response to a stimulus. Starting from the mid–twentieth century, these two views have been combined, leading to a concept of pain as a subjective experience with distinct discriminative and emotional components.

Pain is a social and medical complex problem, as it can lead to serious disability, affect the quality life of affected patients, associate to other pathologies, such as depression, anxiety and sleep disorders. Untreated pain may become self-perpetuating because pain has immunosuppressive effects that leave patients susceptible to other disease (Stucky et al, 2001). Moreover, a condition of persistent pain may lead to inability to work and consequently to relevant social costs.

A recent survey of more than 46,000 people in 16 European countries revealed that almost 1 in 5 adult in Europe suffers from chronic pain – i.e. moderate-to-severe non-cancer pain persisting for more than 6 months (Pain in Europe Survey). Italy was the third country with the highest prevalence (26%, n=3,849) with just over 1 in 4 adults reported suffering from chronic pain (Pain in Europe Survey). The prevalence of chronic pain increases dramatically with age and at least every second person is affected among people older than 65 years (Societal Impact of Pain).

Effective treatments that address pain and comorbidities can enhance outcome for patients with chronic pain. At the moment, even the most effective therapies may not provide complete pain relief in many patients with chronic pain, and only a better understanding of the mechanisms underling this disorder may improve patients' quality of life.



1. PAIN

1.1 Definition and Classification

The International Association for the Study of Pain has published a definition of pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 2011). Although we use a single word to describe any feeling that is unpleasant and hurts, this does not mean that pain is a confined entity.

There are different classifications of pain. For instance, pain can be classified according to its nature in **nociceptive**, **inflammatory** and **pathological**.
Nociceptive pain is what we feel when we touch something too hot, cold, or sharp and is defined as "the neural processes of encoding and processing noxious stimuli (Loeser and Treede, 2008). It is a protective mechanism consequent to activity produced in the peripheral and central nervous system by stimuli that have the potential to damage the organism. This activity is produced by nociceptors that can detect mechanical, thermal and chemical changes above a set threshold (Basbaum and Jessel, 2000). There are two major classes of nociceptors. The first includes medium diameter myelinated afferents (Aδ) that mediate acute, localized, fast pain. These fibres differ from the Aβ fibres that are rapid conducting fibres of larger diameter activated by innocuous mechanical stimuli. The second class of nociceptors includes small diameter unmyelinated fibres (C) that are activated by poorly localized or slow pain (Meyer et al, 2008) (Fig. 1.1). Electrophysiological studies have led to Aδ nociceptors classification into two main classes:

- Type I (HTM: high-threshold mechanical nociceptors), responding to both mechanical and chemical stimuli; these nociceptors are sensitized in the presence of tissue injury;
- Type II that have a much lower heat threshold than type I, but a very high mechanical threshold.

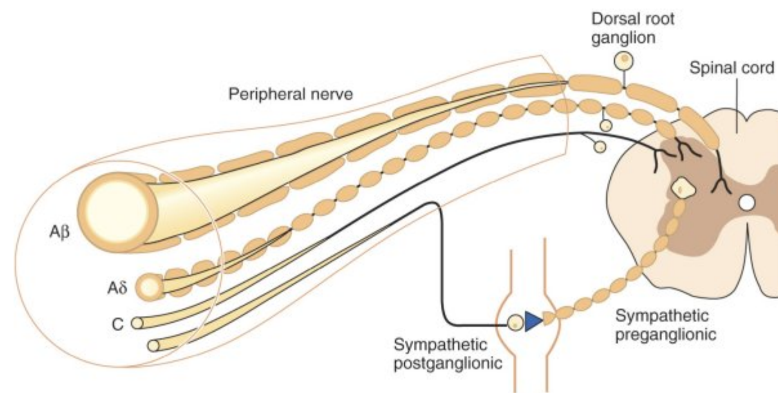


Fig. 1.1: Components of a typical cutaneous nerve. There are two distinct functional categories of axons: primary afferents with cell bodies in the dorsal root ganglion, and sympathetic post-ganglionic fibers with cell bodies in the sympathetic ganglion. Primary afferents include those with large-diameter myelinated (Aβ), small-diameter myelinated (Aδ), and unmyelinated (C) axons. All sympathetic postganglionic fibers are unmyelinated. (Picture adacted from Mcgraw.hill).

The activation of this nociceptors and, hence of nociceptive primary afferent neurons, results within seconds in a transient, well localized pain that does not require long-term changes. Primary sensory neurons have the roles of transduction of noxious but not low-intensity peripheral stimuli, conductions of action potential to the central nervous system (CNS) and transmission to central neurons (Woolf and Costigan, 1999) (Fig. 1.2).

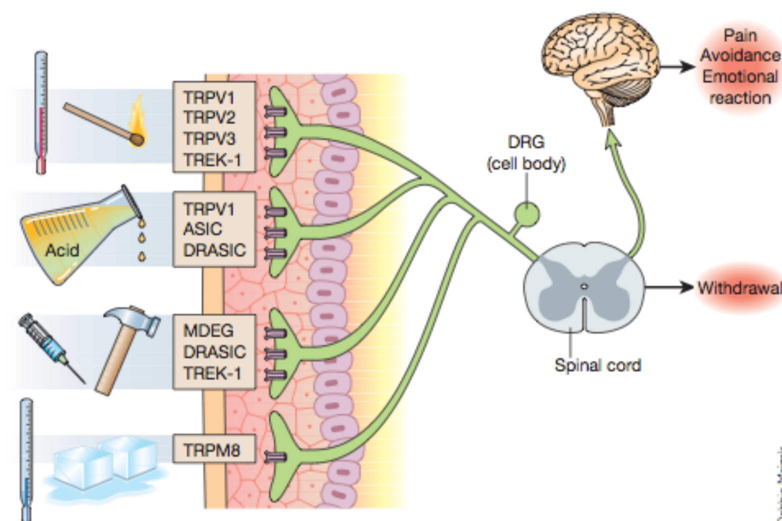


Fig 1.2: Nociceptive pain. Noxious stimuli are transduced into electrical activity at the peripheral terminals of unmyelinated C-fiber and thinly myelinated Aδ-fiber nociceptors by specific receptors sensitive to mechanical stimuli, heat, protons and cold. This information is then conducted via the spinal cord in the CNS to the cortex where the sensation of pain is experienced. (Picture adapted from Scholz and Woolf, 2002).

Inflammatory pain is a protective attempt by the organism to remove injurious stimuli and to initiate the healing process (Woolf, 2010). It is caused by activation of the immune system by tissue injury or infection. Immune cells are activate both in the periphery and within the CNS in response to tissue damage, inflammation or mechanical nerve lesions (Watkins et al, 2001). Tissue damage is often accompanied by the accumulation of endogenous or infiltrate factors released from activated nociceptors or non-neural cells that reside within the injured area. All these factors are usually referred as the "inflammatory soup", consisting of different molecules including neurotransmitters, peptides, eicosanoid and lipids, cytokines and chemokines. The interactions between receptors expressed on the surface of nociceptors and these substances enhance the excitability of the nerve fiber, and enhance their sensitivity to temperature and mechanical stimuli (Basbaum et al, 2009) (Fig. 1.3).

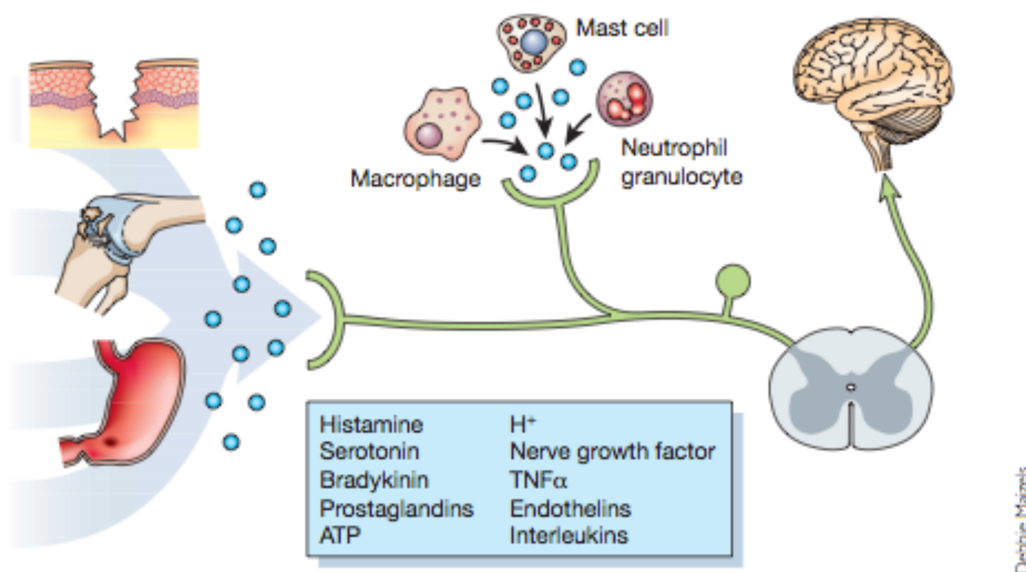


Fig. 1.3: Inflammatory pain. Damaged tissue, inflammatory and tumor cells release chemical mediators creating the "inflammatory soup" that activates the nociceptors afferents. Following this activation, there is the activation and then major responsiveness of neurons in the CNS. Picture adapted from Scholz and Woolf, 2002).

Pathological pain is, instead, a consequence of an abnormal functioning of the nervous system. Pathological pain is a complex disease state of the nervous system

usually occurring after damage to the nervous system like in the case of neuropathic pain but also in conditions in which there is no such damage or inflammation (dysfunctional pain) (Woolf, 2010) (Fig. 1.4).

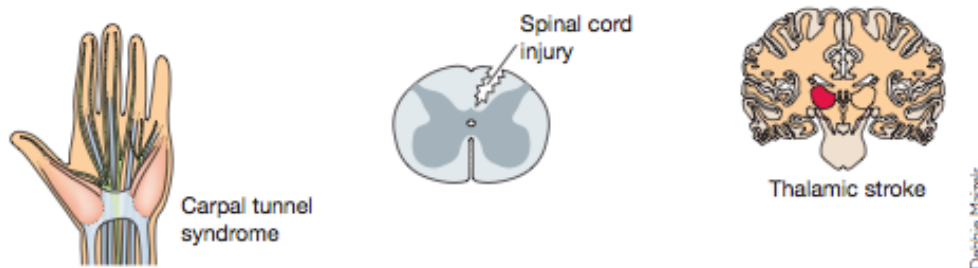


Fig. 1.4: Some conditions leading to neuropathic pain. This pain is a consequence of a lesion or dysfunction of the nervous system. It can be caused by condition affecting the central and peripheral nervous system. (Picture adapted from Scholz and Woolf, 2002).

In the 1994 the IASP defined neuropathic pain as a "pain initiated or caused by a primary lesion or dysfunction in the nervous system". This definition was revised in the 2008 in "Pain arising as direct consequence of a lesion or disease affecting the somatosensory system". This revised definition fits into the nosology of neurological disorders. The reference to the somatosensory system was derived from a wide range of neuropathic pain conditions ranging from painful neuropathy to central post-stroke pain (Loeser and Treede, 2008).

Peripheral neuropathic pain is different from other types of pain for the injury or permanent loss of primary afferent fibres, usually indicated as deafferentation. Positive sensory phenomena, like spontaneous pain, allodynia and hyperalgesia (Box 1) are characteristic of patients with neuropathic pain and could have many underlying mechanisms, including ectopic generation of impulses or *the novo* expression of neurotransmitters and their receptors and ion channels. Direct injury to central structures may permanently alter sensory processing and cause central neuropathic pain. However, the mechanisms underlying this last type of pain are still unclear.

BOX 1

ABNORMAL PAIN SENSITIVIY

ALLODYNIA

"Pain due to a stimulus that does not normally provoke pain" (IASP, 2008).
It is common experience that the touch of the skin affected by sunburn is painful: this is an example of allodynia as touch is a tactile stimulus that in normal conditions does not evoke pain. Allodynia is a clinical feature of many painful condition, such as neuropathies, complex regional pain syndrome, postherpetic neuralgia, fibromyalgia, and migraine.

HYPERALGESIA

"Increased pain from a stimulus that normally provokes pain" (IASP, 2008).
Primary Hyperalgesia refers to pain felt on the site where the stimulus is applied and is due to the release of prostaglandins and painful metabolites. Local anaesthesia of the area can abolish the pain response.
Secondary Hyperalgesia is the pain felt in an area adjacent to or remote of the site of injury. This state is not caused by sensitization of nociceptive nerve endings but is solely due to change in the processing of sensory information in the central nervous system.

A few important anatomical considerations need to be made to better understand the mechanisms of neuropathic pain.

i) First, the location of the damage along the peripheral nerve is responsible for the different intensity in the response and for different types of neuroplasticity (*wind-up*, *long term potentiation* (LTP) and *long term depression* (LDP)). These processes are different if they are positioned at the level of the peripheral terminations, along the nerve, close to the ganglion, at the dorsal root or at the central endings in the spinal cord (Lembeck , 1983; Coggeshall et al. , 1980).

ii) Second, the different peripheral nerves, such as those present in the skin, in the joint, in the muscle or in the visceral tissue, possess different types of fibres and different proportions of somatosensory polimodal nerve fibres (Lawson, 1996), and therefore show different capacity to respond to injury.

iii) Finally, the anatomical location of pain can diversify from the distribution of the damaged nerve, like, for example, the occurrence of contralateral symptoms like in the case of an unilateral damage (Bennett, 1994).

Electrophysiological studies have shown that ectopic activity could be generated at any anatomical regions that mediate the sensory experience. Growing evidence for peripheral neuropathic pain, however, points to substantial ectopic activity arising in primary neurons. After peripheral nerve damage, spontaneous activity is generated at multiple sites, including in the site of injury where the axon grown is aborted (neuroma), in the cell body of injured dorsal root ganglia (DRG) neurons (Amir et al, 2005) and in the close intact afferents (Wu et al 2002). Voltage-gated sodium channels contribute largely to the generation of ectopic activity, in fact the use of local anaesthetics produce a robust inhibitory effects (Sheets et al, 2008). The continuous spontaneous activity, with the opening of the sodium channels, increases the release of aspartate and glutamate at the level of the dorsal horn of the spinal cord, and decreases the release of GABA (Woolf et al, 1998). An important role seems to be played by the activation of NMDA receptors, in fact drugs that attenuate central sensitization by acting on calcium channel subunits to decrease transmitter release and on NMDA channels to reduce transmitter action are effective treatment options in neuropathic pain (Dworkin et al, 2007).

Pain can also be classified according to its duration in **acute** and **chronic**. **Acute pain** is the body's normal response to damage such as a cut, an infection, or other physical injuries. This type of pain usually comes on fast and often goes away in no more than a few weeks or months if treated properly. Chronic pain, instead, generally refers to intractable pain that exists for three or more months and does not resolve in response to treatment (Grichnik and Ferrante, 1991).

Finally, pain can be classified according to the clinical conditions it can be associated to, such a painful peripheral neuropathies (e.g. phantom pain, posttraumatic neuralgia, herpes zoster and postherpetic neuralgia, diabetic neuropathy), central pain syndromes (e.g. stroke, multiple sclerosis, spinal cord injury), complex painful neuropathic disorders (complex regional pain syndromes) and mixed-pain (combination of nociceptive and neuropathic pain, e.g. chronic low-back pain) (Baron, 2009).

BOX 2

DIFFERENT CLASSIFICATION OF PAIN

PAIN INTENSITY

Can be broadly categorized as mild, moderate and severe. It is common to use a numeric scale to rate pain intensity where 0 = no pain and 10 is the worst pain imaginable:

- Mild: ≤ 4/10
- Moderate: 5/10 to 6/10
- Severe: ≥ 7/10

PAIN DURATION

- Acute pain: pain of less than 3 to 6 months duration
- Chronic pain: pain lasting for more than 3-6 months. Or persisting beyond the course of an acute disease or after tissue healing is complete.
- Acute-on-chronic pain: acute pain flare superimposed on underlying chronic pain.

1.2 Anatomy of pain

As previously mentioned, nociceptors are a very important element in the detection and transmission of painful sensations. The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) and have both a peripheral and central axon branch to innervate the target organ and the spinal cord, respectively.

Primary afferent nerve fibres project to the dorsal horn of the spinal cord, which is organized into distinct laminae (Basbaum and Jessel, 2000). In 1952, the neuroscientist Redex divided the dorsal horn of the spinal cord of cat in six Laminae based on the difference of the size and density of neurons (Redex, 1952). Afterwards, this subdivision was applied at other species, including human, monkey and rats. Laminae I and II, which are often referred to as superficial dorsal horn, are the main target of nociceptive primary afferents. This region is the most important in the transmission of pain and its neural organization is quite known. However, also the deeper laminae have an important role in pain: some primary nociceptive afferents

terminating in this region and many of the neurons present in these laminae are activated as a result of noxious stimulation.
Furthermore, low-threshold afferents which terminate in more internal laminae are, at least in part, responsible of the tactile allodynia (Campbell et al, 1988).
In the Lamina I of the dorsal horn there is an important class of neurons involved in processing of painful information: the projections neurons. These NK1-expressing lamina I neurons make occasional collateral projections to deeper layers (Cheunsuang et al, 2000), but they terminate extensively within the parabranchial area of brainstem with limited termination in the medullary dorsal reticular formation, periqueductal gray area, thalamus and reticular formation (Hunt and Mantyh, 2001).

It is clear, at this point, that dorsal horn neurons are the first station for pain processing. They receive information from primary afferent that innervate the skin and deeper tissues. Primary afferents entering into dorsal horn, form excitatory synapses with neurons using glutamate as main neurotransmitter.
These neurons include:

- the already mentioned projecting neurons, with axons that transmit the information to different areas of the brain
- interneurons, with axons that remain within the spinal cord and contribute to local neuronal circuitry.

The organization of interneurons in the dorsal horn is very complex and little is known about their complex neural circuitries. Intrathecal administration of antagonists either for GABA or glycine receptors may cause allodynia and a light touch of the skin can become a noxious stimulus. This suggests that one of the functions of inhibitory interneurons is to suppress the activity evoked by tactile stimuli so that they are not perceived as painful stimuli (Todd, 2013).

The transport of the nociceptive information to higher levels of the central nervous system occurs through multiple and parallel upward projections that lead the signal from the spinal cord to higher brain centres. Neurons ascend to higher centres via the contralateral spinothalamic and spinoreticular tracts, which are located in the anterolateral white matter of the spinal cord.
The thalamus is the key area for processing of somatosensory information. Axons travelling in the lateral and medial spinothalamic tract terminate in the medial and

lateral nuclei, respectively, and from these area neurons project to the primary and secondary somatosensory cortices, the insula, the anterior cingulate cortex and the prefrontal cortex. These areas play various roles in the perception of pain and also interact with other areas of the brain, as cerebellum and basal ganglia (Basbaum, 2009).

Another important pathway in the process of nociceptive information is the descending tract that has a role in the modulation of pain. Noradrenaline and 5-HT are the key neurotransmitters involved in descending inhibition. The inputs of the pain modulatory circuit branch to different areas, as the hypothalamus, the amygdala, and the rostral anterior cingulate cortex feeding to the midbrain periaqueductal gray region (PAG). Neurons within the nucleus raphe magnus and nucleus reticularis gigantocellularis, which are included within the rostral ventromedial medulla (RVM), have been shown to project to the dorsal horn to directly or indirectly enhance or diminish nociceptive traffic, influencing the experience of pain (Field et al, 2005) (Fig. 1.5).

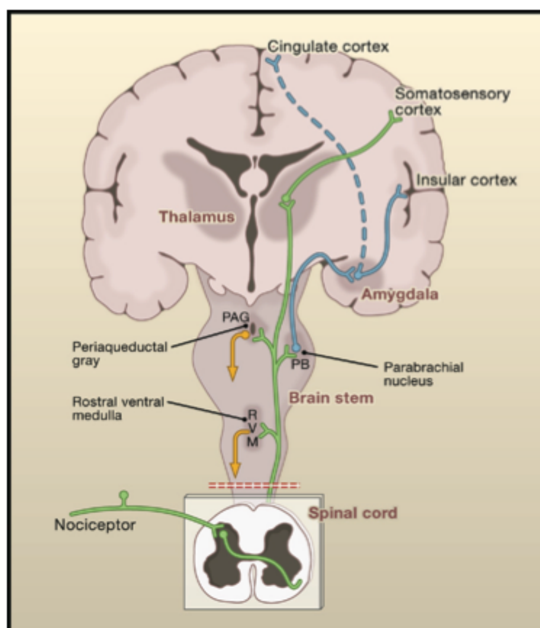


Fig. 1.5: Anatomy of the Pain Pathway. Primary afferent nociceptors convey pain information to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons transmits information to the somatosensory cortex via the thalamus. Other projection neurons engage the cingulate and insular cortices via connections in the brainstem and amygdala (Basbaum et al, 2009)

1.3 Experimental models

Despite the current controversy on whether data from animal models can apply to humans, it is undoubted that this research represents a source of valuable information in many medical areas. Animal models are very important for preclinical studies of neuropathic pain, for testing new drugs and, specially, for investigation to better understand underlying mechanisms.
Over the year, a number of pain models have been developed and characterized, able to model some of the features observed in clinical condition. Some of these models, the more relevant for this thesis, are here described:

- **Neuropathic pain models**

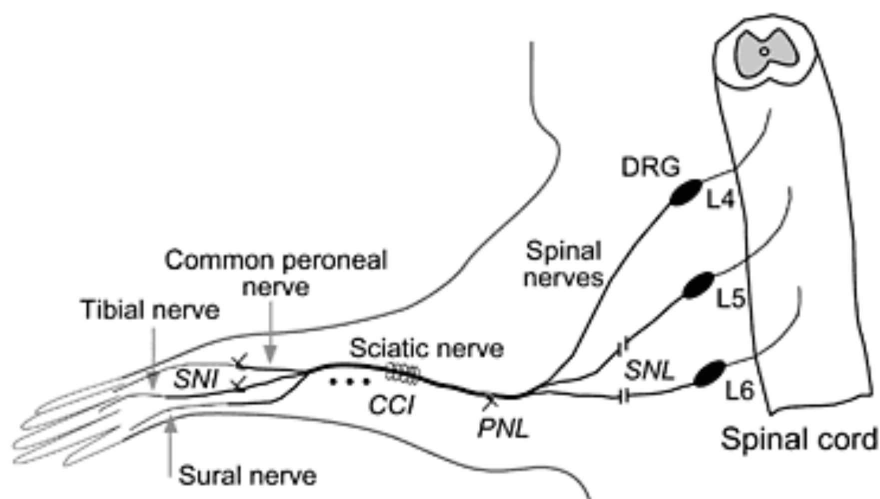


Fig. 1.6: Experimental pain model (See in the text).

✓ **Chronic Constriction Injury** (CCI). This model was developed by Bennett & Xie (1988) and consists of four chromic gut sutures, placed loosely around the common sciatic nerve at intervals of 1-2 mm so that they do not completely impede circulation thought the epineurium (Fig. 1.6). Animals with CCI develop hypersensitivity to light tactile stimuli and thermal hyperalgesia, as suggested by a significant decrease in response latency to noxious radiant heat, but not to noxious mechanical stimuli and cold allodynia. The extreme

hypersensitivity of the animals to cold represent an important feature of this model, in comparison to other models e.g. spinal nerve ligation. However, in this model is not possible to precisely control the relative populations of the different types of afferent fibres that are injured in the procedure.

- ✓ **Spinal nerve Ligation** (SNL). This is the model of traumatic nerve injury that has become the most commonly employed and studied model of neuropathic pain. This model was developed by Chung and colleagues (1992) and involves the tight ligation of L4 and L5 branches of sciatic nerve in rats, and only L5 nerve in mice (Fig. 1.6). These nerves are ligated distal to the DRG with silk suture. Removal of the spinal transverse process is required for an easy access to the spinal nerve (Kim & Chung, 1992). Robust enhanced responses to light tactile and noxious thermal stimuli are present within 1-2 days after surgery, and are persistent for weeks. Tactile allodynia develops also within 1-2 days after injury and is constant for over 50 days (Chaplan et al, 1994). The principal advantage of this model is that spinal nerves are ligated at specific spinal segments. This allows the manipulation of intact nerves and to distinguish the corresponding segments from the un-injured ones. The main disadvantage is the complexity of execution.

- ✓ **Spared Nerve Injury** (SNI). This model is caused by tight ligation and subsequent resection of the common peroneal and tibial nerves, leaving the sural nerve intact (Decosterd & Woolf 2000) (Fig. 1.6). Behavioural signs of neuropathic pain are evident within few days after surgery and are maintained for over 9 weeks. The level of responses appears greater when the stimuli are applied to the receptive field of the sural nerve. A key advantage of this model is that changes in the injured and adjacent uninjured nerves, and in the territories innervated by these nerves, can be studied independently (Decosterd & Woolf 2000).

- **Inflammatory pain models**

✓ **Formalin**

The formalin test was originally described by Dubuisson and Dennis (1977). They described in detail the behaviour induced by formalin and also developed a scheme for pain quantification based on the total time spent in different behavioural states, which were characterized by the animal elevating, licking, biting or shaking the injected paw. This led to the observation of two different phases of nociceptive behaviour in the rat, one phase immediately after formalin injection, lasting for about 5 min, and one phase starting approximately 20 min after the injection and lasting about 40 min. The first phase results from the direct stimulation of nociceptors, whereas the second phase are dependent in part by the central sensitization of spinal cord circuits secondary to the barrage of input that occurs during first phase (Dubuisson et al, 1977; Tjolsen et al 1992). The central or peripheral origin of the second phase has been largely discussed (Tjolsen et al, 1992). Some groups believe that the second phase is a consequence of neuronal activation during the first phase (Coderre et al, 1993). This hypothesis is not supported by experiments in which the blocking of the first phase by substances with rapid actions does not suppress the second phase (Taylor et al, 1995, 1997). Then, the second phase cannot be defined as a consequence of the first, although it originates also from peripheral mechanisms.
The formalin test has been widely used in a variety of studies on the effects of exogenous and endogenous substance (Dubuisson and Dennis, 1977; Hunskaar et al, 1985; Hunskaar et al, 1986; Meunier et al, 1998) on the two different phases.

✓ **CFA**

Arthritis is the inflammation of a joint, which can include infiltration of inflammatory cells, synovial hyperplasia, bone erosion and new bone formation, narrowing of the joint space, and ankylosis of the joint (Bendele et al, 1999). The major complaint by individuals who have arthritis is joint pain (Lane, 1997).
The injection of complete Freund's adjuvant (CFA) induces arthritis. Arthritic pain can be modelled by the intraarticular injection of a suspension of heat-killed *Mycobacterium butyricum* or *Mycobacterium tubercolosum.* This model of chronic arthritis is characterized by joint inflammation, cartilage destruction and bone erosion,

which persist for at least several weeks, and is associated to pain behaviour (Red and Dubner, 1999).

✓ **IL-6 and PGE_2**

Interleukin 6 (IL-6), a pro-inflammatory cytokine, is a significant mediator of nociceptive plasticity in pre-clinical pain model and is implicated in several human pain conditions. It is secreted by T cells and macrophages to stimulate immune responses, i.e. during infection and after trauma, especially burns or other tissue injuries leading to inflammation. Serum IL-6 levels increase significantly in patients immediately after surgery (Holzheimer and Steinmetz, 2000; Notaricola et al, 2011). Furthermore, increased IL-6 has been described in the skin around an incision site (Bryan et al, 2005) as well as in preclinical incision-induced pain models (Sahbaie et al, 2009; Liang et al, 2008). Elegant genetic studies have demonstrated that IL-6's pain promoting qualities are mediated by IL-6 receptors expressed by nociceptors (Quarta et al, 2011).

Prostaglandin E_2 (PGE_2) is a key factor in the generation of an exaggerated pain sensation evoked by inflammation (Vane et al, 1998). A great number of *in vivo* models studies have shown that peripherally injected PGE2 produces hyperalgesia and allodynia both in experimental animal models and in humans (Ferreira et al, 1972; Kuhn and Willis, 1973). This nociceptive effect seems to be related to the ability of PGE2 to sensitize peripheral terminals of small diameter, high threshold, primary afferent fibers to thermal, chemical and mechanical stimuli (Schaible and Schimdt, 1988; Kumazawa *et al.*, 1993, 1996; Mizumura *et al.*, 1993). The intradermal injection of PGE_2 causes an episode of acute hyperalgesia that resolves within few days.

- **Priming model**

The notion that "chronic" pain is a phenomenon distinct from "acute" pain is widely accepted as common sense among both physicians and the lay public (IASP, 1994). Yet, there is no definition of chronic pain that distinguishes it mechanistically from acute pain. Current working definitions of chronic pain, for the most part resort to fixed temporal cut-offs after which point acute pain switches to chronic pain. The

arbitrariness of this approach is amply emphasized by the range of calendar-based periods that are used, for example: 1 month (Magni, 1987), 3 months (Croft et al, 1993), 6 months (IASP, 1994) or 1 year (Brekke et al, 2002) (See BOX 2). Clinical experience, however, suggests that the difference between acute and chronic pain is not arbitrary, and that there can be a functional transition of pain states from acute to chronic (Casey et al, 2008). This transition seems to be associated with a time-dependent disconnection of the generation of pain from the initial tissue injury, and to decreased responsiveness to some therapies that successfully treat acute pain (Biondi, 2006). Alternatively acute pain may persist for long periods without ever undergoing a “chronicization” (Cerbo et al, 2000) of its underlying mechanism.

Understanding the cellular mechanisms underlying chronic pain states is a critical step in the development of new therapies to specifically target the distinct mechanisms of chronic pain. Numerous studies have implicated plastic changes in central nervous system circuitry, driven by increased activity in nociceptive primary afferent nerve fibres (Kayser et al, 1998). However, until recently, very little was known concerning the cellular changes underlying these very long lasting alterations in excitability of primary afferents associated with chronic pain states.

Separating the mechanisms of acute and chronic pain was a key concern in initial investigations that led to the discovery of the phenomenon of hyperalgesic priming. To investigate the clinical observation that some chronic pain conditions can be initiated by one or more transient episodes of acute pain (e.g., complex regional pain syndrome type I, occupational repetitive stress disorders), a protocol was developed to temporally isolate mechanisms that maintain chronic pain from those underlying an antecedent acute pain (Aley et al, 2000) (Fig. 1.7).

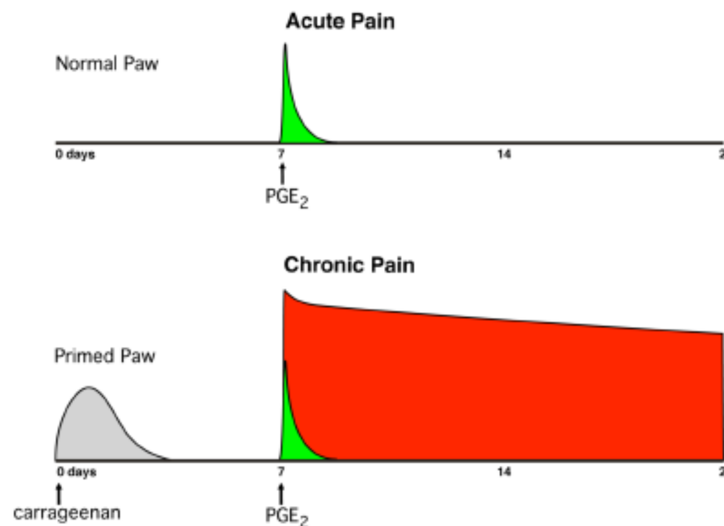


Fig. 1.7: Chronic hyperalgesia associated with inflammation-induced hyperalgesic priming. (A) In the normal (unprimed) paw, a small intradermal injection of prostaglandin E2 (PGE_2) causes an episode of acute hyperalgesia (decreased threshold for paw withdrawal from a pressure stimulus) lasting less than 4 hours. (B) Injection of the inflammogen carrageenen causes an episode of hyperalgesia that lasts less than 4 days (gray-filled curve). After carrageenan-induced hyperalgesia is no longer present, the paw remains in a latent state of hyperalgesic priming. In this state, an injection of PGE_2, which would cause only acute hyperalgesia in the normal (unprimed) paw, now induces an additional chronic hyperalgesia. In comparison to the unprimed paw, this hyperalgesia is greater in magnitude and duration, lasting at least 3 weeks.

A short-lived inflammation is induced in the rat hind paw by intradermal injection of a very low dose of the inflammogen carrageenan. The resultant inflammation, visible as localized redness with minimal swelling, is associated to acute hyperalgesia detected as a decrease in threshold of withdrawal response to a mechanical pressure stimulus applied to the inflamed paw. Both the acute inflammation and the associated hyperalgesia resolve within 4 days, leaving the animal with no signs of ongoing inflammation or hyperalgesia (indeed, carrageenan injection is often used as a model of acute inflammatory pain). However, when the paw is challenged with a new inflammatory stimulus, even weeks later, a dramatically enhanced hyperalgesic response is triggered. Thus, injection of a low dose of an inflammatory cytokine, e.g., prostaglandin E_2 (PGE_2), which in a naïve rat paw would cause only a brief hyperalgesia lasting less than 4 hours, now evokes hyperalgesia lasting at least 24 hours. In addition to this prolongation, hyperalgesic priming also causes an increase in the magnitude of hyperalgesia: the dose-response relationship for PGE_2-induced hyperalgesia is shifted to the left by more than an order of magnitude (Parada et al, 2005) (Fig. 1.7). Hyperalgesia induced by other endogenous inflammatory mediators,

including serotonin and adenosine are similarly enhanced. This latent hyper-susceptibility to inflammatory hyperalgesia that is still present, undiminished, weeks later, is termed hyperalgesic priming.

1.4 Pharmacology of pain

Strong efforts have been made by clinicians and scientists in understanding the underlying mechanisms of chronic pain disorders and developing affective analgesic therapies. However, in the last years, new molecular targets for potential analgesic development were identified, but only few new drugs have been successfully included in clinical use (Kissin, 2010). Various analgesic drugs are use for the treatment and management of pain, with distinct mechanisms and site of antinociceptive activity (Fig. 1.8). As extensively discussed, pain is a complex disease with a myriad of mechanisms contributing to its modulation. Each of these mechanisms could represent a potential targets for pharmacological intervention. Moreover, there are some classes of analgesic agents that may be more effective than others for specific types of pain (Maizels et al, 2005).

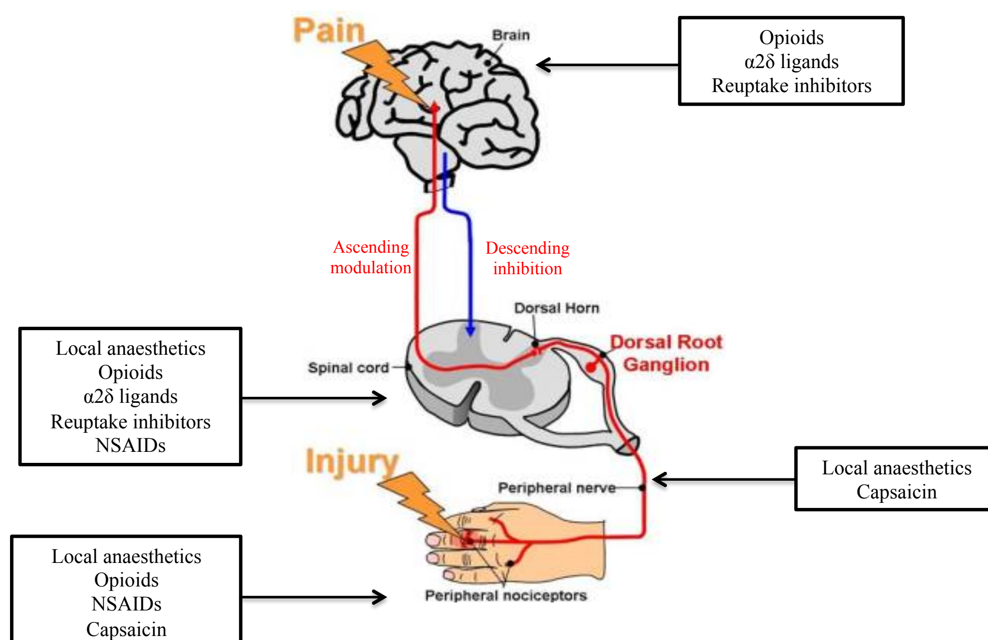


Fig. 1.8: Pharmacology of pain. Drugs commonly used in the treatment of pain act at various levels in the complex network of mechanisms that transmit stimuli from the periphery to the brain

To treat acute pain, due for example to surgery or trauma, conventional analgesics are used in the clinical practice. Opioids and non-steroidal anti-inflammatory drug (NSAIDs) are the first choice in the treatment of this type of pain because they decrease the nociceptive input. Opioids are a mainstay of therapy for the management of moderate to severe nociceptive pain (Practice guidelines for chronic pain management, 1997; Practice guidelines for acute pain management, 2004), which is pain caused by activation of peripheral afferent terminals by noxious thermal, chemical or mechanical stimuli (Woolf, 2004). Opioids act through the activation of μ-opioid receptor in the CNS that can inhibit afferent nociceptive impulse transmission (Einspahr et al, 1980; Feng et al, 2012). Moreover, the activation of μ-opioid receptor within the midbrain and the brainstem facilitates descending inhibitory pathways or suppress descending facilitatory pain pathways (Inturrisi, 2002). Nevertheless, opioid’s effect is also associated with some side effects, such as nausea, vomiting, constipation and pruritus (Kalso et al, 2004; Wheeler et a, 2002), that may affect the patients' compliance (Kalso et al, 2004). NSAIDs are the most commonly used especially for the treatment of acute and chronic musculoskeletal and post-surgical pain (Argoff, 2002). This class of analgesic acts inhibiting the enzyme cyclooxygenase, which thereby reduces the production of inflammatory mediators, including the prostaglandins (Argoff, 2002). NSAIDs are also used as monotherapy for mild to moderate pain. Most of the new analgesic drugs developed in the last years belong to these categories (Kissin, 2010). In the case of chronic pain, the available pharmacological therapies offer palliative symptom control but are not curative. Also, pharmacological therapy is usually based on a comprehensive assessment and part of a multimodal and multidiscipline treatment plan. For example, currently therapy for neuropathic pain is based on tricyclic antidepressant and antiepileptic drugs, the most frequently studied drug classes (McQuay et al, 1996; Laughlin et al, 2002). Opioids and analgesics are a second-line choice (Anderson, 1999).

Recognition of similarities between neuronal hyperexcitability in epilepsy and neuropathic pain led to exploration of antiepileptic drugs as neuropathic pain therapies. The use of certain anticonvulsant drugs, such as the $\alpha_2\delta$ ligands, in the management of neuropathic pain has been extensively studied in such condition as painful diabetic neuropathy and postherpetic neuralgia (Collins et al, 2000). These agents are known to bind the $\alpha_2\delta$ subunit of voltage-dependent calcium channels

within neuronal membranes (Joshi and Taylor, 2006; Gee et al, 1996), which is thought to inhibit the release of excitatory neurotransmitter by pre-synaptic neurons (Joshi and Taylor, 2006). Furthermore, $\alpha_2\delta$ ligands may activate descending inhibitory pathways, stimulating the increase of spinal norepinephrine concentration (Tanabe et al, 2005; Hayashida et al, 2007). Starting from these observations led to the approval of gabapentin and pregabalin for neuropathic pain.

In the lasts years, results obtained from clinical trials, and also observation of direct patient care suggested that use of a single pharmacological agent for the treatment of chronic pain states is able to provide a 30% reduction in pain intensity (Argoff, 2011; Dworkin et al, 2007). As said before, pain is a complex disease and there a re a variety of physiological mechanisms involved. Multidrug analgesic approaches take advantage of complementary mechanisms of different drug classes to enhance analgesia and/or reduce side effect. The association of different classes of analgesic agents is common and has been integrated into the clinical practice for the treatment of both acute and chronic pain (Dworkin et al, 2007; Practice guideline for acute pain management, 2004). Important progress is being made in dissecting out the molecular and cellular mechanisms that operate in sensory pathways to generate those neural signals that we ultimately interpret as pain. Much of currently available clinical treatment is only partially effective and may be accompanied by distressing side effect or have abuse potential. So, to develop effective treatments, it is necessary to investigate new pathways to better understanding the mechanisms involved in its pathogenesis.

2. AUTOPHAGY

2.1 Definition and classification

The correct balance between the production and destruction of macromolecules and organelles is very important for the maintenance of cell homeostasis. Eukaryotic cell can use two different systems to degrade their cellular components: the ubiquitin proteasome system (UPS) and the lysosome. The UPS exclusively degrades proteins, mainly short-lived ones, that have to be tagged by ubiquitin in order to be recognized by the proteasome (Ciechanover et al, 2000). The lysosomal system, instead, is responsible for degrading macromolecules, including proteins, and for the turnover of organelles by autophagy (Mizushima et al, 2008).

The term autophagy, coined by the biologist Nobel-laureate Christian de Duve, is derived from the Greek words *auto* (self) and *phagein* (to eat) and refers to the major intracellular degradation system by which cytoplasmic components are delivered to and degraded by the lysosome. Autophagy can be classified in: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy (Klionsky, 2005) (Fig. 2.1). **CMA** allows the direct lisosomal import of unfolded, soluble proteins. A chaperon protein binds first to its cytosolic target substrate and then to a receptor on the lysosomal membrane where the unfolding of the protein occurs. The unfolded cytosolic target protein is then translocated into the lysosome for its degradation (Chen and Klyonsky, 2011). In the case of **microautophagy**, the cytoplasmic material is directly engulfed on the organelle's surface by membrane rearrangement (Wang and Klionsky, 2004). **Macroautophagy** is thought to be the major type of autophagy and is characterized by the formation of cytosolic double-membrane vesicles called autophagosomes. Here, we refer to macroautophagy simply as "autophagy".

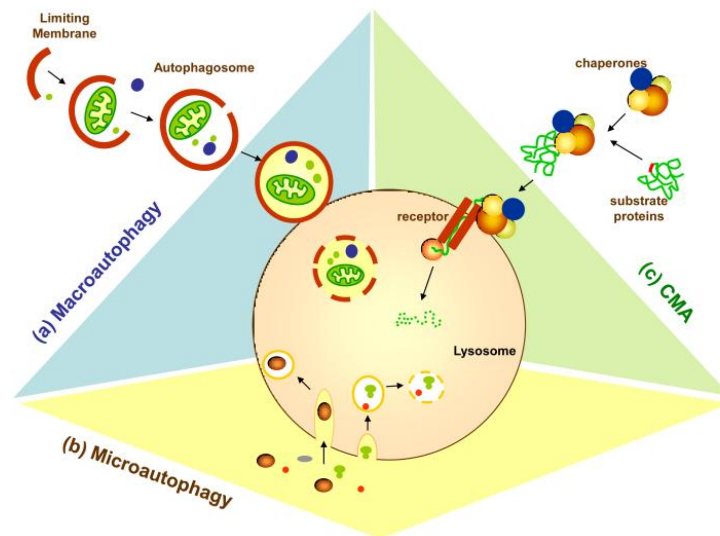


Fig. 2.1: Types of autophagy in mammalian cells. Three different main types of autophagy have been described in mammalian cells: (a) Macroautophagy, (b) Microautophagy and (c) Chaperone-mediated autophagy (CMA). (Cuervo, 2010).

Autophagy was first identified in the 1960s in mammalian cells. However, its molecular mechanisms have only recently begun to be elucidated. One breakthrough in the understanding of the molecular basis of autophagy was achieved by the identification of the genes involved in this process, which in yeast are termed autophagy-related genes (ATG) (Nakatogawa et al, 2009). The identification of Atg genes allowed for the assessment of the importance of this pathway in various contexts, as well as for a more detailed dissection of its mechanism of action.

The signals that regulate autophagy are diverse. Autophagy is highly inducible, with starvation and other stresses able to rapidly increase the number of autophagosomes. Autophagy induction in response to starvation is mediated in part via inactivation of the mammalian target of rapamycin (mTOR) and activation of Jun N-terminal kinase (JNK), while energy loss induces autophagy by activation of AMP kinase (AMPK). Also other pathways involving for example calcium, cyclic AMP, calpain and the inositol triphosphate (IP3) receptor can modulate autophagy (Metcalf et al, 2012).

2.2 A multi-step process

Autophagy is a highly dynamic, multi-step process. Like other cellular pathways it can be modulated at several steps, both positively and negatively.

Autophagosome formation

Membrane dynamics during autophagy are highly conserved from yeast to plants and animals. In the first step of autophagosome formation, cytoplasmic constituents are sequestrated by a membrane called phagophore or isolation membrane. Complete sequestration by the elongating phagophore results in formation of autophagosome, which is typically a double-membrane organelle. This step is a simple sequestration of cytoplasmic components, and no degradation occurs yet.

Among the 31 Atg proteins, 18 (Atg 1-10, Atg 12-14, Atg 16-18, Atg 29 and Atg 31) are involved in autophagosome formation and are called "AP-Atg proteins" (Klionsky et al, 2003).

The Atg1 complex is an essential component for autophagy induction acting downstream of the multiprotein TOR complex 1 (TORC1) (He and Klionsky, 2009). TORC1 inhibition by rapamycin stimulates autophagy in the presence of nutrients, suggesting that TOR downregulates autophagy (Noda et al, 1998). The nucleation and assembly of the initial phagophore membrane requires also the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which is composed of the PtdIns3K Vps34 (vacuolar protein sorting 34), a myristoylated serine/threonine kinase Vps15 (p150 in mammalian cells), Atg14 (Barkor or mAtg14 in mammalian cells) and Atg6/Vps30 (Beclin 1 in mammalian cells) (He and Klionsky, 2009). Class III PI-3 kinase, notably Vps34 (vesicular protein sorting 34) is involved in various membrane-sorting processes in the cell, but is selectively involved in autophagy when complexed to Beclin-1 and other regulatory proteins (Baker, 2008). Vps34 is unique amongst PI3-kinase in only using phosphatidylinositol (PI) as substrate to generate phosphatidyl inositol triphosphate (PI3P), which is essential for phagophore elongation and recruitment of other Atg proteins to the phagophore (Xie and Klionsky, 2007). The function of Beclin 1 in autophagy is regulated by BCL-2 (B-cell lymphoma/Leukemia.2) an anti-apoptotic protein that inhibits autophagy by binding and sequestering Beclin 1 under nutrient-rich conditions; dissociation of Beclin from

Bcl-2 is required for autophagy induction (Itakura et al, 2008). Another Beclin 1 partner is UVRAG (UV irradiation resistance-associated gene), which interacts via the coiled-coil region of Beclin 1. UVRAG was shown to be a member of the class III PI3-kinase complex and a positive regulator of autophagy (Liang et al, 2006). Ambra1, a protein containing a WD40 domain that activates Beclin-1 also regulates autophagy and has a crucial role in embryogenesis (Fimia et al, 2007).

During the elongation step, the Atg12-Atg5-Atg16 complex localizes on the isolation membrane and elongates to engulf cytoplasmic components. In the later stage of elongation, the complex progressively dissociates from the isolation membrane. Finally the isolation membrane closes to form the autophagosome (maturation step).

Autophagosome maturation and degradation

Studies in yeast and mammals have identified two ubiquitin-like proteins, Atg12 and Atg8/LC3 and their conjugation systems that are proposed to act during elongation and expansion of the phagophore membrane. Atg12 is conjugated to Atg5 in a reaction that requires Atg7 and Atg10 (E1 and E2-like enzymes, respectively). The Atg12–Atg5 conjugate then interacts non-covalently with Atg16L, which oligomerizes to form a large multimeric complex called the Atg16L complex (Fig. 2.2 A). Atg8/LC3 is cleaved at its C terminus by Atg4 to generate the cytosolic LC3-I with a C-terminal glycine residue, which is conjugated to phosphatidylethanolamine (PE) in a reaction that requires Atg7 and the E2-like enzyme Atg3 (Fig. 2.2 B). The lipidated form of LC3, named LC3-II, is attached to both sides of the phagophore membrane, but is ultimately removed from the autophagosome outer membrane. This step is then followed by the fusion of the autophagosome with a late endosome/lysosome (Rubinsztein et al, 2012).

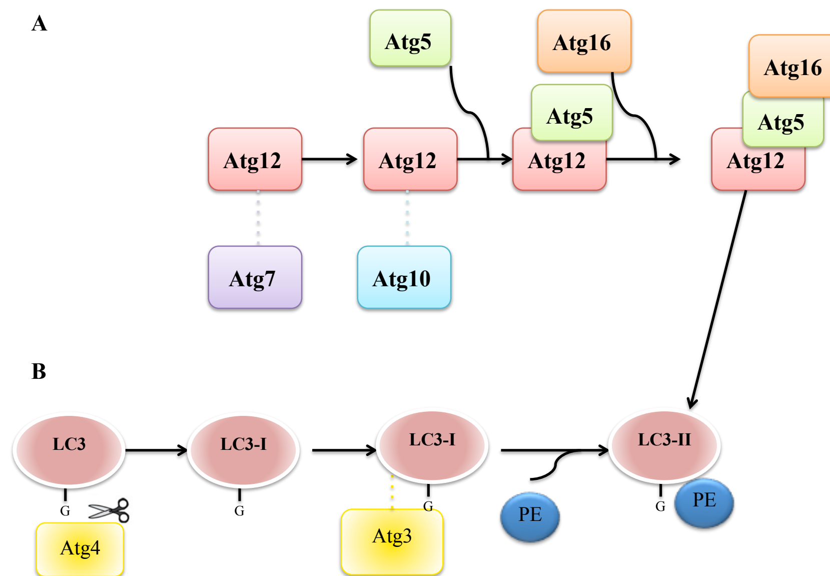


Fig. 2.2: Atg12 and LC3 conjugation systems involved in autophagosome formation. The elongation and shape of the autophagosome are controlled by two protein (and lipid) conjugation systems, similar to the ubiquitin systems: the ATG12 and LC3 (also known as ATG8)–phosphatidylethanolamine (PE) conjugation pathways, which include E1-activating and E2-conjugating enzymes. ATG12 is initially conjugated to ATG7 (an E1-activating enzyme) and then is transferred to the E2-like conjugating enzyme ATG10 (**A)**. This intermediate presents ATG12 for conjugation to an ATG5 lysine residue. The ATG5–ATG12 conjugate, stabilized non-covalently by ATG16, triggers oligomerization on the outside membrane of the growing autophagosome, and enhances LC3 carboxy-terminal lipidation through the LC3 conjugation system **(B)**.

LC3 is one of the best characterized proteins involved in autophagosome formation and LC3-II is regarded as a reliable autophagosome marker. LC3 is synthesized as proLC3, which is cleaved by Atg4B to form LC3-I, with the carbox terminal Gly exposed (Kabeya et al, 2000). LC3-I is activated by Atg7, transferred to Atg3, and finally conjugated to PE. The carboxy-terminal Gly of LC3 is also essential for the formation of the thioester bond with Cys residues in the active site of Atg7 and Atg3, and for the formation of an amide bond with PE (Sou et al, 2006). These two forms of LC3 can be distinguished because of their different mobility during SDS-PAGE: the soluble form LC3-I, located in the cytosol, migrates slower, while LC3-II linked to the membrane migrates faster (Fig. 2.3). Increased levels of LC3-II, consequent to an increased reaction of lipidation, are generally considered an indicator of autophagy induction (Klionsky, 2012).

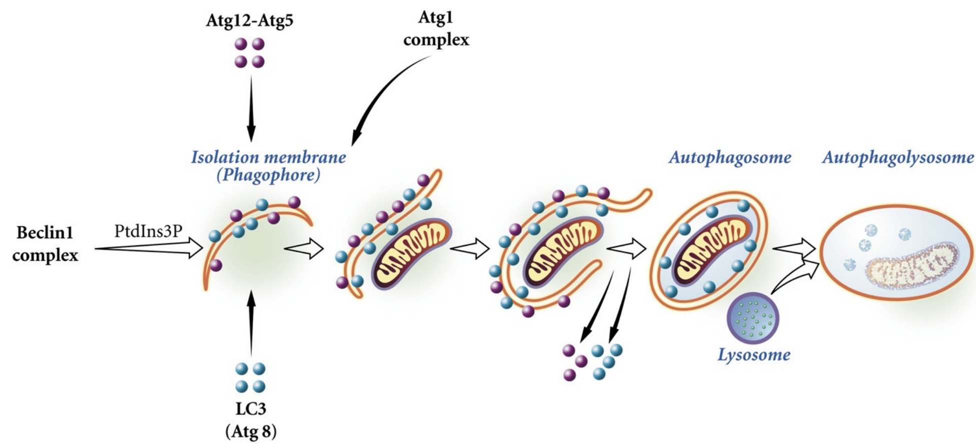


Fig. 2.3: Autophagosome formation (from Pattingre et al, 2008).

After autophagosome-lysosome fusion, the outer membrane of autophagosome is incorporated into the lysosome/vacuole. The process of degradation consists of two steps: a first breakdown of the autophagosome membrane to deliver its contents into the lysosome lumen, and the following degradation by the various enzymes contained in the lysosome/vacuole. The breakdown and degradation process are dependent on acidification of the vacuol lumen (Nakamura et al, 1997) and on a series of lisosomal/vacuolar acid hydrolases, including proteinases A and B and the lipase Atg15 in yeast (Fass et al, 2006; Teter et al, 2001) and cathepsin B, D and L in mammalian cells (Tanida et al, 2005). The small molecules resulting from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular function under starvation conditions. All these steps are of fundamental importance for the flux of material through the autophagic pathway (Codogno and Meijer, 2005). Any blockade in the maturation of autophagosomes, fusion with lisosomal compartment or impairment of the lisosomal function or biogenesis would result in an accumulation of autophagosomes that would inevitably slowdown or interrupt the autophagic flux (Rubinsztein et al, 2009).

An important autophagic substrate: p62

The sequestosome 1/SQSTM1 or p62 is a cellular protein ubiquitously expressed and conserved in animals but not in plants and fungi (Moscat et al, 2009). Different signalling pathways including cell stress, survival/death and inflammation are mediated by p62 (Moscat et al, 2009). This protein directly interacts with LC3 on the

isolation membrane through the LC3-interacting region and is characterized as an autophagy substrate (Fig. 2.4). Because p62 is localized on the autophagosome via LC3-interaction and is constantly degraded by the autophagy-lysosome system, impairment of autophagy results in its accumulation. Komatsu's group showed the molecular mechanism linking autophagy, p62, and inclusion body formation. They observed that genetic ablation of p62 suppressed the appearance of ubiquitin-positive protein aggregates in hepatocytes and neurons, suggesting an important role of p62 in inclusion body formation observed in various neurodegenerative disease (Komatsu, 2007).

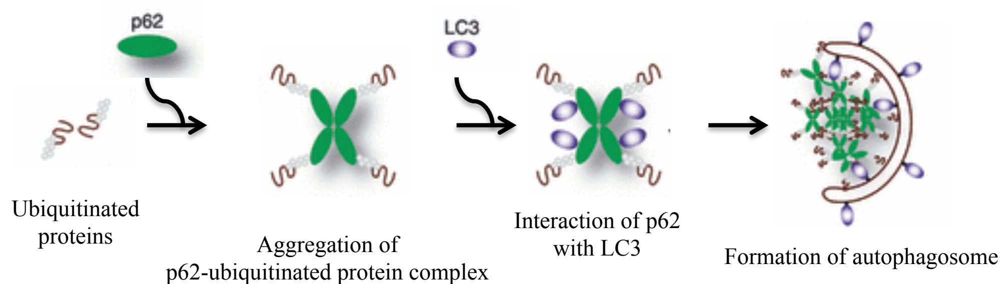


Fig. 2.4: Interaction p62 and LC3 (figure adapted from Komatsu and Ichimura, 2010)**.**

Also, p62 is considered to act as a receptor for ubiquitinated proteins, organelles, and microbes, which it sequesters into the autophagosome (Johansen and Lamark, 2011) and is involved in several signal transduction pathways. Indeed, p62 functions as a signalling hub that may determine whether cells survive by activating the TNF receptor-associated factor 6 (TRAF6)-NF-kB pathway, or die by facilitating the aggregation of caspase-8 and downstream effector caspases (Moscat, 2009). On the other hand, p62 regulates the transcription factor Nrf2, whose target genes include antioxidant proteins and detoxification enzymes (Komatsu et al, 2010). The protein p62 interacts with Nrf2-binding site on Keap1, a component of Cullin3-type ubiquitin ligase for Nrf2. This interaction stabilizes Nrf2 and activates the transcription of Nrf2 genes (Komatsu et al, 2010). Therefore, excessive p62 accumulation or aggregation might lead to hyperactivation of these signalling pathways.

2.3 Physiological role of autophagy

Starting from the discovery of the key role of autophagy in the cellular homeostasis, growing attention in recent years has been focussed on the study of this biological event. Autophagy plays a crucial role in different cell functions and its regulatory mechanisms are not an isolated process, but are insert in a complex network and are coordinated with other cellular activities to maintain cell homeostasis. A better understanding of autophagic network and its role in human disease may make it as a potential target for therapeutic intervention.

Autophagosome formation occurs at a basal rate in most cells as main system for quality check of cytoplasmic content and control of cell homeostasis (Ravikumar et al, 2010). Stimulation of autophagy during periods of starvation is an evolutionarily conserved response to stress in eukaryotes (Yang and Klionsky, 2010). Indeed, under starvation conditions, the degradation of proteins and lipids allows the cell to adapt its metabolism to the environmental changes and to meet its new energy needs. The stimulation of autophagy plays a major role at birth to maintain energy levels in various tissues after the maternal nutrient supply via the placenta ceases (Kuma et al, 2004). Starvation-induced autophagy is cytoprotective by blocking the induction of apoptosis upstream of mitochondrial events (Boya et al, 2005). Autophagy is also essential during development and differentiation. The pre-implantation period after oocyte fertilization is dependent on autophagic degradation of components of the oocyte cytoplasm (Tsukamoto et al, 2008). Autophagy remodelling of the cytoplasm is involved during the differentiation of erythrocytes, lymphocytes and adipocytes (Ravikumar et al., 2010). Autophagy is also crucial for the homeostasis of immune cells contributing to the regulation of self-tolerance (Nedjic et al., 2008) (Fig. 2.5).

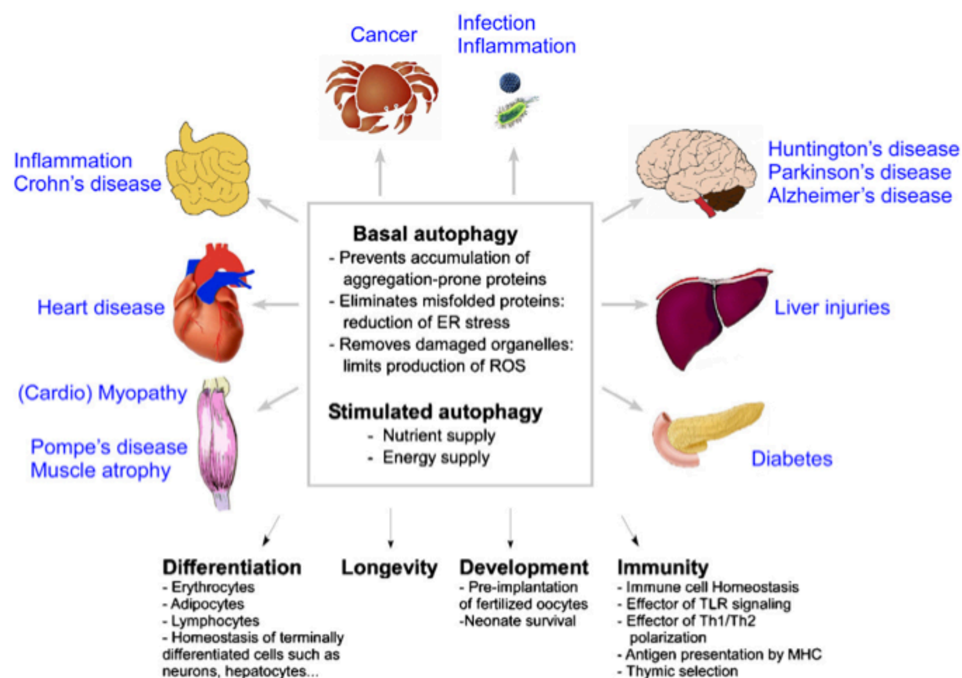
One of the most exciting aspects of autophagy is its connection with aging and health span (Cuervo, 2008). The proteolytic activity decrease with age and it has been associated, at least in part, with the accumulation of damaged cellular components in almost all tissue of aging organisms (Cuervo et al, 2005). Indeed, results obtained with caloric restriction (Jia et al, 2007; Morck and Pilon, 2006), suggested that restoration of autophagy in older animals is practicable and it might help to retard progression of aging.

2.4 Pathological role of autophagy

As previously largely discussed, autophagy is crucial for cell adaptation and survival under extreme condition. Moreover, autophagy is not an isolate event, but works together with different process to keep a correct homeostasis, essential for cell survival. Any perturbation of this balance can influence cell fate.

Basal autophagy is important as a housekeeping process to prevent the accumulation of defective organelles (i.e. mitochondria) and/or aggregation-prone proteins in the cytoplasm. It is not clear in which case autophagy is cytoprotective or cytotoxic. The absence of autophagy may increase cell death during metabolic stress, but by contrast, excessive levels of autophagy may promote cell death, presumably by self-cannibalization. This mechanism was proposed for cancer: autophagy might enhance the survival of rapidly growing cancer cells (Levine, 2007). Autophagy plays an important role also in infectious disease for the elimination of pathogens like bacteria, viruses and parasites and thus contribute to the innate immunity (Levine and Deretic, 2007). In neurodegenerative diseases, indeed, including Huntington's, Alzheimer's and Parkinson's disease, the observation of accumulation of protein aggregates in the brain suggested the involvement of autophagy in the pathogenesis of these disorders (Ravikumar et al., 2010). But, recent studies revealed that the stimulation of autophagy limits the accumulation of toxic products and protects neurons against degeneration. In a model of Alzheimer Disease (AD), the process of autophagy was found to be dysregulated, and the treatment with rapamycin abolished the AD pathology and reversed memory deficits (Barnet and Brewer, 2011). In one recent study, Russo and colleagues provide evidence of the autophagy impairment in a model of glaucoma, suggesting a protective role of autophagy in retinal ganglion cells. The authors demonstrated that excitotoxicity negatively regulates autophagy through the calpain-mediated cleavage of Beclin-1 (Russo et al, 2011). The pathological role of autophagy in aggregation-prone proteins diseases is strengthened by a recent study showing that a drug that enhances autophagy promotes the degradation of mutant, aggregation-prone α1-antitrypsin in the liver, and consequently reduces hepatic fibrosis (Hidvegi et al., 2010). Also in muscle diseases autophagy play an important role in the clearance of aggregation-prone proteins. Blockade of the autophagic pathway leads to the cardiomyopathy and myopathy of Danon disease (Ravikumar et al., 2010). Autophagy is also involved in muscle

atrophy but it is unclear whether autophagy has a beneficial effect by promoting survival during catabolic conditions, or a detrimental effect by causing atrophy. In the heart, basal autophagy is necessary to maintain cellular homeostasis and is upregulated in response to stress in hypertensive heart disease, heart failure, cardiac hypertrophy, and ischemia-reperfusion (Nakai et al., 2007). In the pancreas, autophagy is required to maintain the architecture and function of pancreatic β-cells (Ebato et al., 2008). Defective hepatic autophagy probably makes a major contribution to insulin resistance and to predisposition to type-2 diabetes and obesity (Yang et al., 2010) (Fig. 2.5).



Adapted form Beau et al, 2011

Fig. 2.5: Physiological and pathological roles of autophagy.

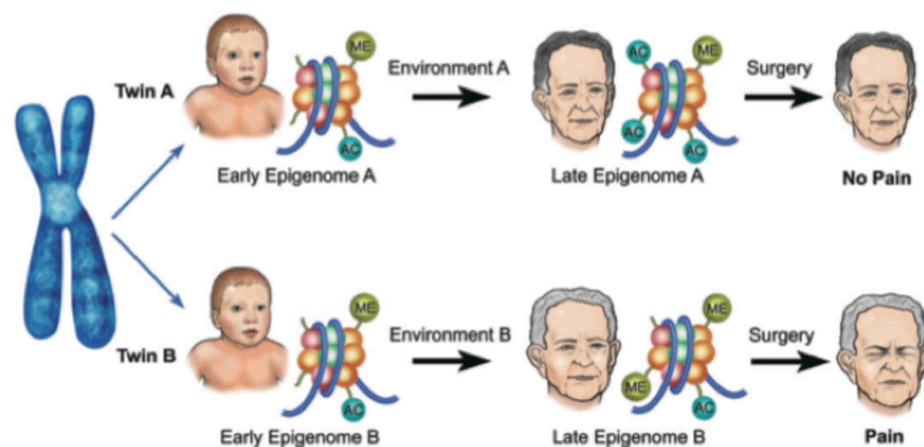
Emerging evidence suggest the involvement of autophagy also in the pathophysiology of chronic pain. Our group showed for the first time that basal autophagy is disrupted in a model of neuropathic pain (Berliocchi et al, 2011). Along this line, other groups confirmed this observation strengthening the possibility that that modulation of autophagy may represent a new target in the treatment of neuropathic pain (Shi et al, 2013, Marinelli et al, 2013; Zhang et al, 2013).

3. EPIGENETIC MECHANISMS

3.1 Regulation of gene expression in pain states

In recent years, more insight has been gained on the cellular mechanisms that link inflammation, peripheral sensitization and pain. In addition, there is increasing evidence about the genetic human code (Schuler, 1996) and mutation that are associated with specific chronic pain syndromes (Yuan et al, 2011).

Human studies have shown that monozygotic twins may exhibit significantly different inflammatory and chronic pain phenotypes, indicating that the etiological basis of these disorders cannot simply lay on the individual genetic asset. Indeed, it is now accepted that response to injury is determined by complex interactions between the genome and the environment. These alterations might be epigenetic, namely heritable modification that are not intrinsic to the genetic code, but that affect gene expression in a tissue-specific manner, resulting in an observable phenotype (Fig. 3.1) (Villenueve, 2011).



(from Buchheit et al, 2012)

Fig. 3.1: Epigenome and chronic pain. Twin A and twin B demonstrate similar "epigenomes" at birth with few (if any) differences in methylation and acetylation patterns. Environmental factors throughout development affect histone acetylation and cytosine methylation patterns, resulting in phenotypic differences in adulthood. With surgery or nerve injury these epigenetic differences may result in different predisposition to chronic pain.

Nondevelopmental epigenetic modifications are also triggered by environmental, nutrition and stress (McEwen et al, 2012), and may be playing a role in the onset of chronic pain following nerve injury (Kiguchi et al, 2011; Uchida et al, 2010).

Environmental factors alter gene expression and phenotype for painful disorders by inducing epigenetic modifications (Doehring et al, 2011). Following injury, expression of transcription factors such as nuclear factor-kappa B (NF-kB) is increased (Ma et al, 1998), sodium channels in the injured axon are upregulated (Jin et al, 2006), μ-opioid receptors in the dorsal root ganglion are downregulated (Porreca et al, 1998), substance P expression is altered (Ma et al, 1998), and the dorsal horn of the spinal cord is structurally reorganized through axonal sprouting (Okamoto et al, 2001). Multiple candidate gene association studies have been used for the investigation of pain. A few candidate genetic polymorphisms have been linked to pain susceptibility, including for instance the catechol-O-methyltransferase (COMT) gene. Its gene product modulates nociceptive and inflammatory pain and has been linked to temporomandibular joint pain syndromes (Diatchenko et al, 2005). The SCN9A gene has also been studied as a marker for pain sensitivity. Mutation in this gene, which codes for the alpha-subunit of a voltage-gated sodium channel Na_v 1.7, are known to result in alteration of pain perceptions (Nassar et al, 2004). However, these physiologic and genetic advances do not fully explain why one patient develops chronic pain following an injury, and another patient does not. Despite recent technical advances in acute pain management, 30-50% of patients still develop chronic pain following surgeries (Kehlet et al, 2006).

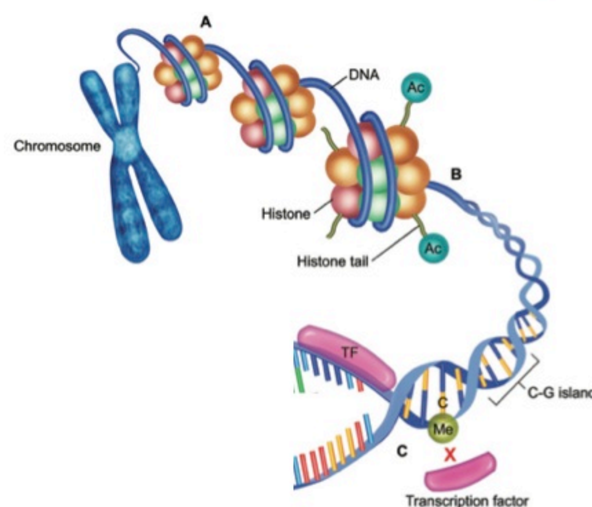
A patient's gene expression profile changes rapidly in the post-injury period, with over 1,000 genes activated in the dorsal root ganglion alone after nerve injury (Hammer et al, 2010). There is significant evidence for epigenetic control of this gene activation from acute to chronic pain. First, immunologic response and inflammatory cytokine expression are under epigenetic control (Hashimoto et al, 2009). Second, glucocorticoid receptor (GR) function, which affects pain sensitivity, inflammation and the development of autoimmune disease, is modulated both through posttranslational mechanisms and DNA methylation (Turner et al, 2008). Third, genes such as the glutamic acid decarboxylase 64 one, coding for pain regulatory enzyme in the CNS, are known to be hypoacetylated and downregulated in inflammatory and nerve injury pain states (Zhang et al, 2011). Finally, epigenetic modifications are

involved in opioid receptors regulation and function, with implications for endogenous pain modulation systems and pain severity (Nielsen et al, 2009).

3.2 Epigenetics

The epigenome sits at the interface of the cellular environment and the genome, enabling epigenetic changes to exert robust control over transcriptional processes. In somatic cells, epigenetic influences are responsible for cellular differentiation and the perpetuation of the cellular phenotype over time and across cell division (Feinberg, 2007; Reik, 2007). All of the cells of a multicellular organism are derived from a single cell, the zygote; therefore they carry identical genetic information. In spite of this, they follow different developmental pathways and differentiate into more than 100 different cells types as found in the human body. The cellular fate and phenotype is determined by epigenetic regulatory mechanisms that are hereditable through cell division and function without changing the DNA sequence (Watson et al, 2008).

The term epigenetics refers to processes that lead to stable and/or heritable changes in gene function without any concomitant DNA sequence changes. Epigenetic modifications regulate the compaction of chromatin and include a variety of facets, the most important being DNA methylation (Fig. 3.2) and post-translational modifications of histones (Fig. 3.2) (Géranton, 2012).



(adapted from Buchheit et al, 2012)

Fig. 3.2: Epigenetic mechanisms. (A) DNA wraps around histone octamer to form a nucleosome, the fundamental building block of chromatin; (B) Histone proteins may be modified through several processes, including acetylation; (C) Methylation of cytosine nucleotides in CG rich sequence prevents the binding of transcription factors and generally silences gene expression.

Over the past ten years, the understanding of epigenetic mechanisms has significantly increased as a result of many seminal studies, such as the discovery of histone demethylases (Shi et al, 2004; Tsukada et al, 2006) and work on the genome-wide distribution of acetylation and methylation marks in human cells line (Ernst et al, 2011; Barski et al, 2007).
There has also been a surge in research investigating epigenetic mechanisms in the fully developed nervous system with a significant literature on memory and synaptic plasticity. Unlike a number of cytoplasmic protein modifications, epigenetic mechanisms possess a number of features that are consistent with a molecular storage device for long-term memory. First, these modifications are believed to be relatively stable in comparison to other alterations. Secondly, this class of modifications is capable of altering gene expression directly, and is therefore able to modulate gene programs known to be involved in learning and memory (Day and Sweat, 2011).

3.3 Epigenetics modifications

Chromatin is the structural conformation of DNA in association with assembly proteins. The nucleosome is the fundamental repeating unit of chromatin, consisting of about 140 base pair of DNA wrapped around a histone octamer (Fig 1.15A).

- **DNA methylation**

DNA methylation was first discovered in calf thymus DNA by Hotchkiss in 1948 (Hotchkiss, 1948). Today it is clear that DNA methylation is a key epigenetic process involved in the control of gene expression (Miranda and Jones, 2007), regulation of parental imprinting (Delaval and Feil, 2004) and stabilisation of X chromosome inactivation (Heard, 2004), as well as maintenance of the genome integrity through protection against endogenous retroviruses and transposons (Howard et al, 2008). It is also implicated in the development of the immune system (Fitzpatrick and Wilson, 2003) and in cellular reprogramming (Reik, 2007), as well as in brain function and behaviour (McCarthy et al, 2009). DNA methylation, which occurs at CpG dinucleotides within DNA (Fig 3.2 C), is catalysed by two different forms of DNA methyltransferases (DNMTs), both of which are expressed in mature neurons (Bird, 1999; Feng et al., 2010).

The eukaryotic DNMT family has five members: DNMT2, DNMT3A, DNMT3B, DNMT3L and DNMT1 (Fig. 3.3). DNMT3A and DNMT3B are *de novo* methyltransferases, whereas DNMT1 is involved in the maintenance of DNA methylation (Margot et al, 2003; Bestor, 2000). Mammalian DNMTs contain at least three structural regions, namely: the N-terminal regularory domain, which is responsible for the localization of DNMTs in the nucleus; the C-terminal catalytic domain, which resembles that of the prokaryotic enzyme; and the central linker, which consists of repeated GK dipeptides (Araujo et al, 2001). For the methylation reaction, S-adenosylmethionine serves as methyl donor. The result is an incredibly strong reaction which has the ability to restore itself if lost. For example, even if one methyl group is somehow removed from one strand, maintenance DNMTs will recognize this and re-establish a methylated cytosine. This peculiar arrangement highlights an important feature of DNA methylation, which is that despite ongoing events that may remove the methylation mark, a substrate exists to effectively perpetuate that mark through time. In fact, this reaction has been proposed to serve as one of the mechanisms that underlie lifelong inactivation of one X chromosome in females (Chow & Brown, 2003; Chow et al,2005). Thus, this reaction has the same basic form as CaMKII phosphorylation, but has been observed to persist across time and despite ongoing cellular stimulation (Day and Sweatt, 2011).

Interestingly, in addition to undergoing methylation in response to environmental stimuli, it appears that demethylation of cytosine bases in DNA may be as or even more important to cellular function (Wu and Zhang, 2010). The mechanisms through which this occurs is presently unclear and controversial. Given that the methyl group and cytosine base at a methylated CpG are linked via a covalent carbon-carbon bond, it is unlikely that an enzyme directly removes the methyl group. However, a number of alternative methods have been proposed that may occur for rapid DNA demethylation (Ma et al, 2009a; Ma et al, 2009b; Wu and Zhang, 2010). Recently, Rao and colleagues suggested that a direct demethylation by proteins, in particular the protein Tet1, and possibly also other Tet family members, are responsible for the conversion of the 5mC into 5hmC (Tahiliani et al, 2009). However, multiple mechanisms have been implicated in demethylation of DNA, including oxidative demethylation, complete excision of the methylated cytosine by DNA glycosylases, and deamination by RNA editing enzymes (Wu and Zhang, 2010). Thus, although the

exact mechanisms underlying demethylation of DNA remain controversial, it is clear that decreases in methylation can play an important role in brain function.

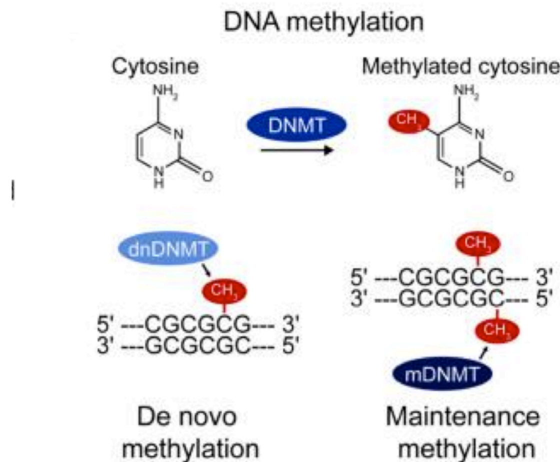


Fig. 3.3: De novo methylation and maintenance methylation

DNMT1 is a major maintenance DNA methyltransferase

DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern during replication. During the replication of eukaryotic genomic DNA, approximately 40 million CpG dinucleotides are converted into the hemimethylated state in the newly synthesized DNA strand. These hemimethylated Cpg sites must be methylated precisely to maintain the original DNA methylation pattern. DNMT1 is located at the replication fork and methylates newly biosynthesized DNA strand directly after the replication round (Hermann et al, 2004).

The structure of DNMT1 indicates the DNMT1 gene could have been formed during the fusion of a prokaryotic DNMT gene with a mammalian DNA binding protein gene (Araujo et al, 2001). Mammalian DNMT1 is also composed of at least three major structural elements. The first 621 amino acids of the N-terminus are not essential for DNMT1 activity (Pradhan and Esteve, 2003). However, the N-terminal DNMT domain is essential for discrimination between hemimethylated and unmethylated DNA strand and is responsible for a decrease in *de novo* methylation activity. The charge-rich motif of then N-terminal domain of DNMT1 interacts with DNMT1 and represses transcription without the participation of HDAC (Rountree et al, 2000). The DNMT1 N-terminal domain can also interact with other proteins, including the proliferating cell nuclear antigen (PCNA), inhibitor of cyclin-dependent kinases

(p21WAF1), E2F1 transcription factor, HDAC1 or HDAC2 (Pradhan and Esteve, 2003; Hermann et al, 2004). The interaction of DNMT1 with numerous protein suppressors of promoters suggests that this DNA methyltransferase is also a crucial element of the transcription suppression complex (Turek-Plewa and Jagodzinski, 2005). The primary structure of human DNMT1 suggests that the entire catalytic site of this enzyme is composed of 500 amino acids and is located at the C-terminal domain (Pradhan and Esteve, 2003). The C-terminal catalytic domain of DNMT1 is characterized by the presence of 10 conserved amino acids motifs. Five of this motifs, namely I, IV, VI, VIII, X, are involved in the binding of substrate to DNMT1 (Araujo et al, 2001).

Recent work by Géranton's group (Tochiki et al, 2012) showed that the mRNA levels of DNMTs are modulated in the superficial dorsal horn during persistent pain states, suggesting that the state of chromatin compaction is regulated during the maintenance phase of chronic pain state. In particular, an overall bilateral up-regulation and an ipsilateral downregulation of DNMTs has been described 7 days after CFA and 7 days after SNI surgery, respectively (Tochiki et al, 2012).

- **Histone modifications**

Histone octamers and their surrounding DNA form a nucleosome, the fundamental building block of chromatin (Fig 3.2 A). Most of the histone complex is inaccessible, but the N-terminal tail protrudes from the nucleosome and is therefore subject to additions that change the three-dimensional chromatin structure and subsequent gene expression (Zhou et al, 2011). Indeed, the N-terminal histone tails may be modified by more than 100 different posttranslational processes including acetylation, phosphorylation and methylation (Fig 3.2). Acetylation is one of the most common modifications. Histone acetyl transferases add acetyl groups altering the histone protein structure. In fact, this change prevents the chromatin from becoming more compact, thus allowing transcription factor to bind more easily. This state of increased acetylation and "permissive chromatin" generally increases transcription activity and RNA production within that genetic sequence, especially when located in gene promoter regions (Struhl, 1998). Conversely, histone deacetylases (HDACs) remove acetyl groups from histones, generally suppressing gene expression. In

concert, these opposite activities serve important regulatory functions (Fig. 3.4) (Buchheit et al, 2012).

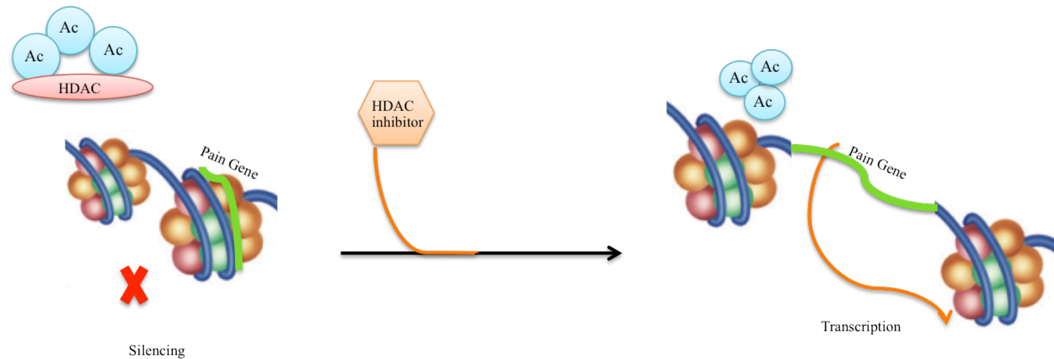


Fig. 3.4: Schematic illustration of HDAC role in gene transcription

3.4 Epigenetic gene regulation in pain processing

Three epigenetic processes have so far been uncovered that can influence expression of nociceptive genes in chronic pain state. These are histone acetylation, DNA methylation and gene transcription via specific factors like REST. Pharmacological interference with the process of histone acetylation can affect pain behaviour, with both systemic and intrathecal administration of HDAC inhibitors having analgesic effects in models of inflammatory pain (Chiechio et al, 2010; Bai et al, 2010). In one study, this effect was shown to be mediated by changes in the expression of the mGluR2 receptor in both DRG and spinal cord (Chiechio et al, 2009). Conversely, a pathological pain state may be able to induce changes in histone acetylation at relevant pronociceptive gene. Injection of an inflammatory agent (complete Freund's adjuvant, CFA) into the paw of rats was shown to lead to transcriptional downregulation of GAD65 in the dorsal raphe nucleus coupled with hypoacetylation at its promoter. The same was true after spinal nerve ligation, which is used to mimic a neuropathic pain state (Zhang et al 2011).

Similar influences on expression could be shown in the case of DNA methylation and its reader molecule MeCP2. The methyl binding protein MeCP2 has been shown to promote abnormal upregulation of a group of genes in inflammatory pain conditions. In rats, its usually repressive function appears to be curtailed through phosphorylation

after injection of CFA into the ankle joint (Géranton et al, 2007). This mechanism was shown to be partly dependent on intact descending serotoninergic input into the spinal dorsal horn (Géranton et al, 2008). Further supporting this role for MeCP2 are studies demonstrating altered pain threshold as a result of reduced MeCP2 expression levels (Samaco et al, 2008). Moreover, two recent reports have emerged as the first to directly measure changes in DNA methylation at genes associated with chronic pain condition (Tajerian et al, 2011; Viet et al, 2011). Finally, there is evidence for the involvement of REST in chronic neuropathy. REST is a transcription factor that recognizes a specific promoter sequence (RE-1 element) present in nearly 2000 genes with primarily neuronal function (Bruce et al, 2004). Partial sciatic nerve ligation, a model of neuropathic pain, resulted in a long-lasting increase in expression of this repressive transcription factor in mouse DRG (Uchida et al 2010). Using chromatin immunoprecipitation, it could further be shown that REST promoter binding is directly responsible for reduced expression of several genes known to be relevant for nociceptive processing in the DRG (Uchida et al 2010).

3.5 FKBP5

The gene FKBP5 codes for FK506 binding protein 51, a 51kDa co-chaperon protein of the Hsp90 complex. This gene is located on the short arm of human chromosome 6 and several lines of evidence suggest that this gene is an important functional regulator of the GR-complex. The glucocorticoid receptor (GR) is a member of the ligand-dependent transcription factor family. Upon ligand binding, the receptor undergoes a conformational change, translocates from the cytosol to the nucleus and modulates gene transcription. The process of GR activation, trafficking and subsequent GR action on gene transcription is regulated by a multiprotein complex that assembles around the molecular chaperone heat shock protein 90 (Hsp90) (Pratt et al, 2006). Recent studies have shown that FKBP5 is a key player of the GR-hsp90 complex. The GR is important in the stress hormone system and is important to terminate the stress response after the end of a threat. Dysregulation in this system has been described in stress-related psychiatric disorders and indicated as a long-term consequence of exposure to early life trauma (Holsboer, 2000; Heim et al., 2008).

After stress signal, the hypothalamus releases corticotropin-releasing hormone (CRH) onto the pituitary gland; the pituitary gland releases the adrenocorticotropic hormone

(ACTH) into the bloodstream; this signal to the adrenal glands to release glucocorticoids (CORT) into the blood stream. Finally, activation of the GR receptor by CORT switches off the stress response in the brain.

FKBP5 is a negative feedback regulator of the glucocorticoid receptor, and its expression is activated by the glucocorticoid receptor in response to an elevation of glucocorticoids, the body’s major stress hormones. FKBP5 suppresses GR activity by interacting whit the glucocorticoid receptor protein and decreasing ligand binding and the subsequent translocation of the complex to the nucleus, thereby promoting resistance to glucocorticoids and e prolonging the stress response (Fig. 3.5).

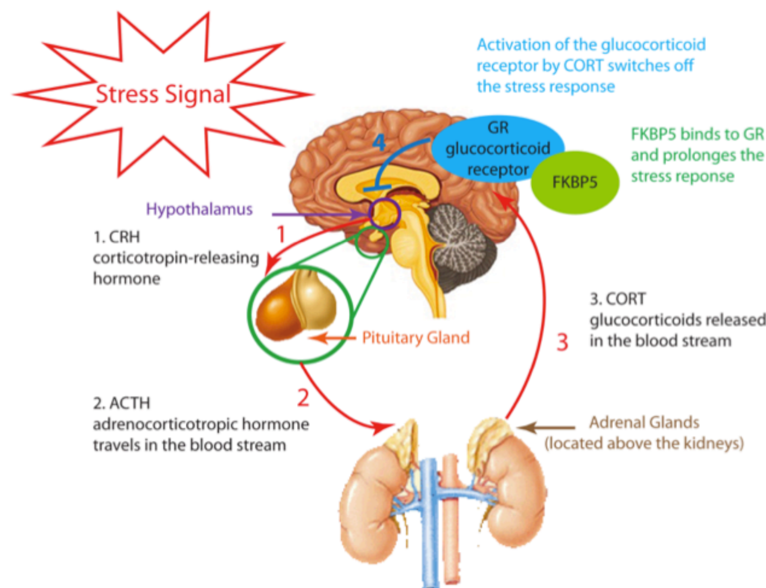


Fig. 3.5: FKBP5 can prolongs stress response by modulating the activity of the glucocorticoid receptor (GR). (Courtesy of Géranton).

There is evidence that single nucleotide polymorphisms (SNPs) in the FKBP5 gene are associated with increased recurrence of depressive disorders (Binder et al, 2004). Variation in one of these SNPs correlates with increased levels of FKBP51. Moreover, increases in FKBP5 in the amygdala produce an anxiety phenotype in mice (O’Leary III et al., 2011). Dickney’s group showed that FKBP5 deletion in mice produces antidepressant behaviour without affecting cognition and other basic motor function (O’Leary III et al., 2011).

- **FKBP5 and its epigenetic regulation**

As already mentioned, epigenetic mechanisms refer to processes that lead to stable and/or hereditable changes in gene function without any concomitant changes in the DNA sequence. Epigenetic changes, especially changes in DNA methylation, have also been reported as long-lasting consequence of early trauma (McGowan et al., 2009), and glucocorticoid receptor activation has been shown to induce local changes in DNA methylation also in the murine FKBP5 locus (Thomassin et al, 2001; Lee et al, 2010). Klengel and colleagues showed that childhood trauma-dependent DNA demethylation in the functional glucocorticoid response element of FKBP5 is linked to increased stress-dependent gene transcription, followed by a long-term disregulation of the stress hormone system and a global effect on brain areas involved in stress regulation (Klengel et al, 2013). They proposed a mechanism to explain gene-environment interaction. They suggest that early life adversity triggers an increase in circulating glucocorticoids (GCs) that act on a Glucocorticoid Response Element (GRE) in a risk allele of FKBP5, in the hippocampus and other tissue. This leads to strong transcriptional activation and to demethylation of a methylated CpG site in the risk allele. This demethylation can happen only during a critical period early in the life and result in a persistent activation of FKBP5. Adult life stress triggers glucocorticoids release, which results in strong activation of demethylated FKBP5, which in turn suppresses the glucocorticoid receptor, resulting in resistance to glucocorticoids observed in post-traumatic stress disorders (PTSD) (Fig. 3.6).

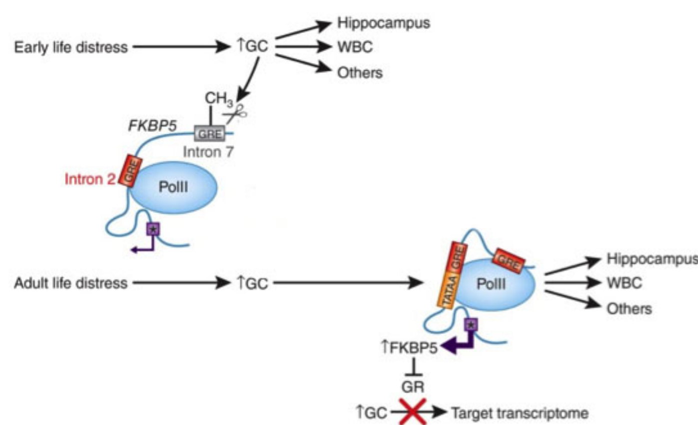


Fig. 3.6: A model mechanism for gene-environment interaction (from Szyf, 2013).

4. AIM

Pain is an important global health problem. Estimates suggest that 20% of adults suffer from pain globally and 10% are newly diagnosed with chronic pain each year (Goldberg and McGee, 2011).

Despite the great advances made in the field of pain research, its complex mechanisms are still largely unknown and the currently available drug therapies are mostly symptomatic and not always effective thus urging new therapeutic strategies. In the past recent years, many efforst have been made to develop better diagnostic tools and to improve our understanding on the biological mechanisms underlying pain and the mechanism of action of analgesic drugs. These efforts are aimed to identify novel therapeutic targets but also to turn existing drugs into more effective and manageable therapeutic tools. On this line, the work of this thesis focused on the characterization of two novel biological processes, **autophagy** and **epigenetic mechanisms,** in experimental models of neuropathic and inflammatory pain, and the study of their role in pain processing.

Autophagy is an active mechanism for degrading damaged organelles and long-lived and unwanted proteins through the autophagosome-lysosome pathway (He and Klionsky, 2009). Growing evidences suggest the involvement of this pathway in neurodegenerative diseases, such as Alzheimer's disease (Nixon et al, 2000), Parkinson's disease (Michiorri et al, 2010) and Huntington's disease (Yamamoto et al, 2006), in which autophagy seems to play a protective role.

Only recently, first by our group then confirmed by others, an involvement of autophagy was also described in the development of pain states. However, observations so far have not allowed drawing definitive conclusions on the role of autophagy in the mechanisms underlying chronic pain.

Epigenetic mechanisms are chemical modifications to chromatin that modulate gene expression without altering the DNA sequence (Géranton, 2012). Among the genes for which there are experimental evidences of their involvement in chronic pain, our attention has focused on the study of the gene FKBP5. Previous studies showed that the expression of this gene is upregulated 2h after painful stimulation (Géranton et al, 2007). This gene is important for the regulation of stress response and has been linked to a range of stress related mental disorders in human (Binder et al, 2008). Growing

evidence support the notion that sensation of pain depends also on physiological factors such chronic stress (Wagner et al, 2013) and this research aimed to create the appropriate experimental tools then to investigate the role of FKBP5 and its regulation by epigenetic mechanisms and stress conditions in pain.

Therefore, the main objectives of this work were:

- To characterize the autophagic process at spinal cord level in different experimental models of neuropathic and inflammatory pain;

- To verify the relevance of spinal autophagy for pain processing;

- To identify pain conditions in which FKBP5 plays a role;

- To study the role FKBP5 on pain processing at spinal cord level;

- To characterize the enzymes involved in DNA methylation

The research work on FKBP5 and epigenetic mechanisms was carried out at the University College London (UCL), under the guidance of Doctor Sandrine Géranton.

5. MATERIAL and METHODS

5.1 Animals

Male Sprague Dawley rats (200-220 g; Central Biological Service, University College London, UK), C57BL/6J male mice (Charles River, Milan) and FKBP5 -/- mice, obtained by Dickey's group (University of South Florida. USA), were used for the experiments. Animals were kept in their home cages at 21°C and 55% relative humidity with a light-dark cycle of 12h (lights on at 08:00 h) food and water were provided ad libitum. All efforts were made to minimise animal suffering and to reduce the number of animal used (Italian D.M. 116/1992 and UK Animal Act 1986).

FKBP5 -/- mice

These mice, obtained by Dickey's, have been generated as published previously (Tranguch et a, 2005). Briefly, by PCR screening to the 129SvJ mouse bacterial artificial chromosome (BAC) library (Genome Systems, St. Louis, MO), BAC clones that contained genomic regions for *FKBP5* were isolated. Restriction fragments were subcloned into pBluescript (pBS; Stratagene, La Jolla, CA) or pZero (Invitrogen, Carlsbad, CA) cloning vectors. The PCR products were amplified from the BAC clones and were then used to construct a targeting vector in the pPGK*neo* vector (a generous gift of James Lee, Mayo Clinic Scottsdale). The targeting vector contained a beta-galactosidase/neomycin cassette flanked by regions homologous to the *FKBP5* gene. Due to the size of the protein it is more practical to partially delete the gene. Thus, when the targeting vector integrates into the chromosome through homologous recombination it removes all of exon 2, which is the first coding exon. Since the only deleted portion of the gene is exon 2 the expression of the beta-galactosidase protein is dependent on the FKBP5 promoter and transcription machinery and expresses in frame with the initiation codon embrional stem (ES) cells were isolated from the 129SvJ mouse and cultured in Knockout DMEM media (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin, essential amino acids, and ESGRO (103 U/ml; Chemicon, Temecula, CA) with irradiated embryonic fibroblast feeder cells. The ES cells were then electroporated at 0.2 kV, 950 μF (Gene Pulser II; Bio-Rad, Hercules, CA) with linearized targeting vectors and selected with G418. DNA from

G418-resistant clones was isolated for Southern blot analysis. A DNA probe was used to distinguish *Pst*I restriction fragments from wildtype allele (~7.5 kb) and targeting vector (~10 kb). Appropriate homologous recombination in ES cell clones was confirmed by PCR using primers complementary to sequences within the neomycin cassette and to 3′ *FKBP5* sequences downstream from the recombination site. ES cell clones containing the targeting vector were injected into C57BL/6 blastocysts and implanted into pseudopregnant 129SvJ females. Chimeric offspring were identified by coat patterns and mated to C57BL/6 mice to obtain germline transmission of the targeting vector. For colony maintenance mice were crossed from C57BL6 onto Swiss-Webster for purposes of fecundity and genetic diversity to be more representative of a human population.

5.2 Inflammatory pain models

- **Formalin**

The formalin test was performed according to Dubuisson and Dennis (1977). Animals were placed in a Plexiglas chamber and allowed to habituate for at least 30 minutes. A mirror was placed below the Plexiglas chamber at a 45° angle to allow for easy viewing of the behavioural response. Mice were gently restrained, and 20 µl of 5% formalin was injected subcutaneously into the plantar surface of the left hind paw using a microsyringe with a 27-gauge needle. Behavioural testing was initiated immediately after formalin injection and maintained for 60 minutes. The incidences of licking and biting were counted at intervals of 5 min.

- **Complete Freud's Adjuvant**

Inflammation was induced by injection of Complete Freud's Adjuvant (CFA; Sigma, UK) at the volume of 10 µl in rats and 5 µl in mice, in the left ankle joint, under isoflurano anaesthesia induced in a chamber delivering 2% isoflurano combined with 100% O2 and maintained during the injection via a face mask. The needle entered in the ankle joint from the anterior and lateral posterior positions, with the ankle kept in plant flexion to open the joint. Sham treatment consisted only of anaesthetizing the animals.

Alternatively, CFA (20 µl) was also injected subcutaneously into the plantar surface of the left hind paw of mice using a microsyringe with a 27-gauge needle. The mice were maintained under isoflurano anaesthesia during the injection.

5.3 Neuropathic pain models

- **Spinal Nerve Ligation**

The spinal nerve ligation (SNL) was performed according to the Kim & Chung model (1992). Under 2% isoflurano anaesthesia, a middle incision was performed in the skin of the back at the L2-S2 levels and the left paraspinal muscles separated from the spinal process at the L4-S1 levels. The left lumbar L6 transverse process was carefully removed to identify the L4 and L5 spinal nerves. The left L5 spinal nerve was isolated and tightly ligated with a 6-0 silk thread (Fig. 5.1). Complete hemostasis was confirmed and the wound was saturated.
The surgical procedure for the sham group was identical to the SNL group, except that the spinal nerve was not ligated.

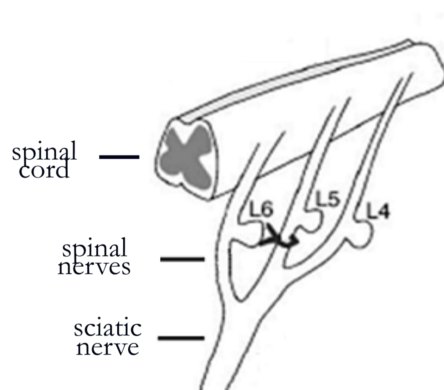


Fig 5.1: Spinal Nerve Ligation model.
The left spinal nerve was isolated and tightly ligated.

- **Spared Nerve Injury**

The spared nerve injury (SNI) was performed as described by Decosterd and Woolf (2000). Under 2% isoflurano anaesthesia the skin on the lateral surface of the thigh was incised and a section made directly though the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves (Fig. 5.2). The common peroneal and the tibial nerves were tight-ligated

with 5-0 silk and sectioned distal to the ligation. Great care was taken to avoid any contact with or stretching of the spared sural nerve. Complete hemostasis was confirmed and the wound was saturated.

For sham control, the procedure involved exposure of the sciatic nerve and its branches without any lesion.

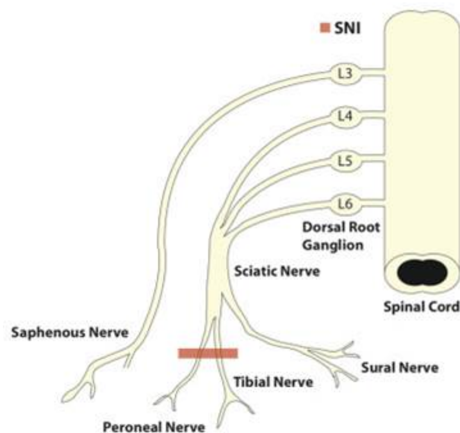


Fig. 5.2: Spared Nerve Injury model.
The common peroneal and the tibial nerves were tightly ligated and sectioned.

- **Chronic Constriction Injury**

Chronic constriction injury (CCI) of the sciatic nerve was performed according to the model described by Bennet & Xie (1988). Under 2% isoflurano anaesthesia, the common sciatic nerve was exposed and dissected from the surrounding connective tissue. Three loosely constrictive ligatures (5-0 silk) were tied around the nerve with a 1-1.5 mm distance between ligatures (Fig. 5.3). The muscle and skin were sutured after complete hemostasis was confirmed. For sham surgery, the sciatic nerve was exposed as described above but no contact was made with the nerve.

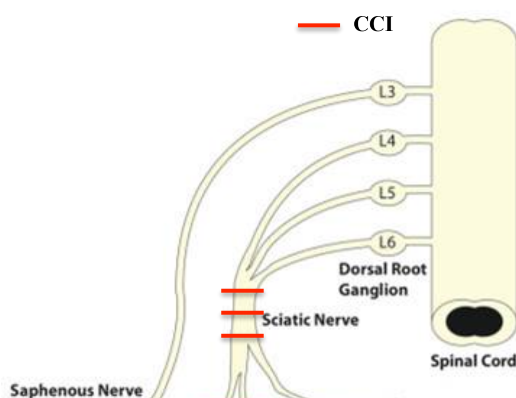


Fig. 5.3: Chronic Constriction Injury model.
Three ligatures were tied around the nerve.

For all the models, after surgery foot posture and general mice behaviour were monitored throughout the postoperative period.

5.4 Induction of chronic hyperalgesic priming

A chronic state of hyperalgesic priming was induced by a first intraplantar injection of 25 µL of IL-6 (0.1 ng). Control mice received 25 µL 0,1% sterile BSA/PBS. Naïve mice received only anaesthesia. Seven days after IL-6 or BSA/PBS injection, mice were injected with PGE_2 (100 ng) in the plantar surface of the left hindpaw in a volume of 25 µL. Allodynia testing was then done at the time points indicated in the figure .

Fig. 5.4: Experimental protocol for hyperalgesic priming

5.5 Behavioural Test

- **Von Frey Test**

Animals were placed in Plexiglas chambers, located on an elevated wire grid, and allowed to habituate for at least 1 hour. After this time, the plantar surface of the paw was stimulated with a series of ascending forces von Frey monofilament (Kim & Chung, 1992; Seltzer et al, 1990; Fuchs et al., 1999). The threshold was determined by using the up-down method as described by Chaplan and colleagues (1994). The up-down method consists in the application of the various filaments according to a sequence that takes into account the type of response of the animal. In particular, the first filament that corresponding to 50% of the threshold of withdrawal in basal conditions is applied. Subsequently, in the presence of pain response, a filament of decreasing strength is applied whereas, in the absence of pain response, a filament to

increasing force is applied. The test continues until at least six measurements are obtained around the value of the 50% threshold of withdrawal. The data were expressed as log of mean of the 50% pain threshold ± SEM.

- **Hargreaves test**

The latency of foot withdrawal to noxious heat stimuli was measured as previously described (Hargreaves et al, 1988). The animals were placed on a glass plate under which a light box was located. A radiant heat stimulus was applied by aiming a beam of light through a hole in the light box to the heel of each hind paw through the glass plate. The light beam was turned off automatically by a photocell when the mouse lifted the foot, allowing the measurement of time between start of the light beam and the foot lift. The withdrawal latency was expressed in seconds.

- **Rotarod test**

Motor performance was evaluated by an accelerating Rotarod apparatus with a 3cm diameter rod starting at an initial rotation of 4 RPM and slowly accelerating to 40 RPM over 100 seconds. Mice were expected to walk at the speed of rod rotation to keep from falling. The time spent on the rod during each of three trials per day was measured and expressed in seconds. Testing was completed when the mouse fell OFF the rod (distance of 12 cm).

- **Acetone test**

Cold sensitivity was evaluated by the acetone test as previously described (Decosterd and Woolf, 2000). Before testing, animals were habituated over a period of 2-3 days by recording a series of baseline measurements. Mice were tested for their paw withdrawal response to a cold stimulus using a drop of acetone applied with a syringe at the center of the plantar surface of a hind paw ipsilateral to the injury. Care was taken to avoid mechanical stimulation of the paw with the syringe. Total time spent in lifting/clutching was recorded with an arbitrary maximum cut-off time of 20 sec.

5.6 Intrathecal administration

This procedure is best applied in awake, conscious mice because the subject's muscle tone and reaction are important contributors to the successful and reliable performing of the injections (Fairbanks, 2003). The mouse was kept firmly, but gently, by the pelvic girdle using thumb and forefinger of the non-dominant hand. This grip should cause the hind legs to splay outward and downward. The skin above the ileac crest is pulled tautly to create a horizontal plane where the needle will be inserted. Using the other hand, the experimenter traces the spinal column of the mouse, rounding or curving the column slightly to open the invertebrate spaces. The needle used was a 30-gauge needle connected to a 10 µl Hamilton syringe and the experimenter attempts to "enter" between the vertebrae. Puncture of the Dura was indicated by a reflexive tail flick. After injection the syringe was rotated and removed and posture and locomotion were checked.

- **Block of lisosomal degradation**

The lisosomal inhibitor chloroquine (Sigma, c6628) was dissolved in a saline solution to have a final 100 µM concentration. The mice were divided into two groups, one treated with the chloroquine solution and the other one treated with the vehicle. Intrathecal injection (i.t.) was performed as described above. A 3 µl i.t. injection was performed every day for three days. After injection the animals were observed and 2h later underwent behavioural test.

- **Silencing of FKBP5**

The *in vivo* silencing of the protein FKBP5 was achieved by using a mouse specific small interference RNA (siRNA) (Thermo Scientific). It is a mixture (SMARTpool) of 4siRNAs that have been modified to help entering the cell. The siRNA was reconstituted in RNase-free H_2O to a final concentration of 2µg/µl. Intrathecal injection of the siRNA solution (2µl/mouse) was performed, as described above, for three consecutive days according to the experimental protocol (See Fig. 7.9 A and 7.10 A).

5.7 Genotype analysis

- **DNA extraction and qPCR**

The DNA was extracted from samples obtained from a small portion of ear tissue from each animal. Each sample was lysed in 75 µl of Alkaline lysis buffer (25mM NaOH, 0.2 mM EDTA, pH12) and heated at 95°C for 30 min. After cooling at +4°C, 75 µl of neutralising reagent (40mM Tris-HCl, pH5) were added to each sample.

The primers used for qPCR are shown in the Table 1. Amplification was obtained starting from 1 µl of DNA and one unit of Taq DNA polymerase (Promega, Madison, USA) in a final volume of 21 µl of amplification buffer. Following an initial DNA denaturation for 4 minutes at 95°C, the amplification was obtained after 40 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 40 sec, followed by a final elongation for 5 min at 72°C. 15 µl of products obtained by amplification were loaded on a 2% agarose gel containing 20 µl ethidium bromide, subjected to electrophoresis and visualized under a UV light (Fig. 5.5).

PRIMERS	Sequence
Forward (for WT and 51KO)	AAAGGACAATGACTACTGATGAGG
Reverse WT	AAGGAGGGGTTCTTTTGAGG
Reverse 51KO	GTTGCACCACAGATGAAACG

Table 1: Sequences of primers used for qPCR

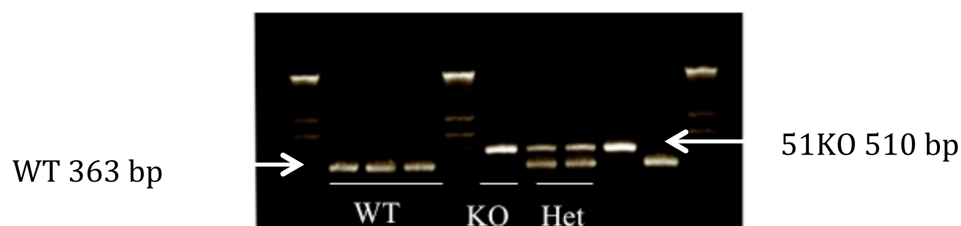


Fig. 5.5 : Mice genotyping. Representative agarose gel used for mice genotyping showing examples of wildtype, heterozygote and knockout mice

5.8 Western blots

For fresh tissue collection, animals were terminally anaesthetized with CO_2 at 7d and 14 d after surgery. The spinal cord segment corresponding to the lumbar area was rapidly removed and the ipsi- and contra-lateral dorsal horn quadrants L4-L5 were dissected out and frozen in liquid nitrogen, then stored at -80°C until further processing. Each sample was homogenised in ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0,1% SDS, 1% IGEPAL, 0,5% Na-deoxyxolate) in the presence of protease inhibitors (Sigma, P8349) and incubated on ice for 40 min. Samples were then centrifuged at 14,000 x g for 115min at 4°C. Total protein content was determined in the supernatants by the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Milan, Italy). For western blot analysis, equal amounts of total proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15%) and transferred onto PVDF membranes (Immobilon-P, Sigma). After blocking for 1 hour at room temperature in Tris-buffer saline containing 0,05% Tween 20 (TBST) and 5% non-fat milk, the membranes were incubate with the primary antibody directed against the protein of interest (Table 2). After several washes, an appropriate HRP-conjugated secondary antibody (goat IgG; Pierce Bio-tecnology, USA) was applied for 1 hour at room temperature. Peroxidase activity was visualized using the ECL Western Blotting Detection kit (ECL, Amersham Biosciences, Italy) and X-ray films (Hyperfilm ECL, Amersham Bioscence). Signal intensity was measured using Fiji software (NIH, USA). For quantitative analysis, the Beclin 1, LC3-I, LC3-II, p62 and α2δ-1 signals of each sample were normalized towards the corresponding GAPDH signal. Changes in signal intensity were then expressed as fold increase of the ipsilateral *versus* the contralateral side for each individual animal.

Antibody	Company and codex	Host	Experimental Conditions
Anti-α2δ1	Sigma D219	Mouse	1:1000; O/N; RT
Anti-p62	MBL PM045	Rabbit	1:1000; O/N; RT
Anti-Beclin1	MBL PD017	Rabbit	1:4000; O/N; RT
Anti-LC3	MBL PM036	Rabbit	1:2000; O/N; RT
Anti-GAPDH	Ambion AM4300	Mouse	1:40000; 1h; RT

Table 2: Antibodies and experimental conditions for western blot analysis

5.9 Immunohistochemistry

For immunohistochemistry, animals were deeply anaesthetized with pentobarbital and perfused transcardially with saline containing heparin followed by 4% paraformaldehyde (PFA) in 0,1 M phosphate buffer (PB; 250 ml/adult rat; 25 ml/adult mouse). The lumbar spinal cord was dissected out, post-fixed in 4% PFA for 2h and then transferred into a 30% sucrose solution in PB containing 0,01% azide at 4°C, for at least 24h. The spinal cord was sectioned on a freezing microtome set at 40 µm thickness. Sections were left to incubate with a primary antibody against the protein of interest (Table 3). Appropriate biotinylated secondary antibody was used at the concentration of 1:400 and left for 90 min. sections were then incubated with avidin biotin complex (ABC Elite, Vector Lab; Vectastain A 1:250 + Vectastain B 1:250) for 30 min followed by a signal amplification step with biotinylated tiramide solution (TSA) (Perkin Elmer, 1:75 for 7 min). Finally, samples were incubated with FITC avidin (1:600) for 2 hour. For double and triple stainings, sections were left overnight at room temperature with the second primary antibody (Table 3). Direct secondary was used at a concentration of 1:500 (Alexa Fluor). All sections were then coverslipped with Fluoromount Aqueous Mounting Medium (Sigma) to protect the fluorescence from fading and stored in dark boxes at 4°C. Controls for immunohistochemistry were included omitting the first or second primary antibodies.

Antibody	Company and Code	Host	Experimental conditions
Anti DNMT-1	Cell Signaling	Rabbit	1:2000* O/N; RT
Anti-GFAP	Dako Z0334	Rabbit	1:4000 O/N; RT
Anti-TET1	EpiGentek A-1020	Goat	1:500* O/N; RT
Anti-cFos	Calbiochem PC38	Rabbit	1:2000* O/N; RT
Anti-NeuN	Millipore MAB377	Mouse	1:1000 O/N; RT
Anti-Iba1	Wako 019-19741	Rabbit	1:1500 O/N; RT
Anti-p62	MBL PM045	Rabbit	1:20000* O/N; RT

*TSA protocol; RT (Room Temperature)

Table 3: Antibody and experimental condition for immunohistochemistry

5.10 Results analysis

- **Confocal microscopy**

All images of double and triple stained tissue were acquired by confocal microscopy using a laser scanning microscope (Leica TSC NT SP). The slides were first viewed under fluorescent microscope and suitable section were chosen based on being spinal cord level L4-L6, an intact superficial dorsal horn and a good p62 and DNMT1 stain. Ranges of 3-9 images were taken per animal. Z stacks of 4 images were taken per section at 0.5 μm intervals. Images were taken for the both ipsi- and contra-lateral side of each section. Laser strength of each scan was not changed throughout any of the imaging.

- **Data analysis and statistics**

We used confocal microscopy images to measure DNMT1 and p62 expression within GFAP, Iba1 and NeuN positive cells. Gain and laser strength were set at the beginning of all experiments and fixed throughout data collection. Cell bodies and their corresponding nuclei were analysed to measure DNMT1 and p62 fluorescent intensity. Analysis to quantify DNMT1 stain in the nuclei of neurons and astrocytes was performed using Fiji software. The anti-NeuN antibody binds to the nucleus and

hence quantification of DNMT1 was possible by using NeuN fluorescence as a mask creating after particle size and threshold were adjusted. The particle size defined the minimum size of neuronal nuclei and DNMT1 nuclei that would be used for analysis, a nucleus smaller than the particle size was excluded and not used in DNMT1 quantification. A threshold was defined as the minimum fluorescence intensity that would be used for further analysis and DNMT1 quantification.

The anti-GFAP antibody binds to an astrocytic skeletal protein hence only astrocytic processes are labelled. Astrocytic nuclei expressing DNMT1 were manually selected by viewing several composite images. If it was not obviously astrocytic or the nuclei appeared incomplete and at the edge of the image or if it was not within the superficial dorsal horn then the DNMT1 nucleus was disregarded.

Fiji Analysis gave three measures of DNMT1 intensity in neurons and astrocytes that were used for statistical analysis. These were: i) average size of DNMT1 nuclei, ii) the mean intensity of DNMT1 expression within the nuclei and iii) the integrated density of DNMT1. Integrated density is a product of average size*mean intensity and gave the best overall representation of changes in DNMT1 expression. There may be an increase in average nucleus size but a decrease in mean intensity if the same volume of DNMT1 is present but in a larger area. Therefore integrated density gives the best representation of intensity of stain per volume of nucleus.

For c-Fos cell counting, the number of immune-positive cells expressing c-Fos was counted in the dorsal horn ipsilateral to the injection. The spinal cord was divided in superficial (I-II lamina) and deeper laminae (III-IV laminae) and the counting was performed in these areas. A range of 3-6 images was taken for each animals and the mean of counting across these section was used for statistical analysis.

For western blot analysis, normalized signals were compared in control and treated animals by a Student's *t* test.

For the behavioural experiments, statistical analysis was performed on the raw or normalized data. The significance of any differences is sensitivity was assessed using repeated-measures two-way ANOVA (SPSS software, Chicago, IL). In all cases, "time" was treated as "within subjects" factors and "genotype" or "treatment" was treated as a between subjects factor. ANOVA tests the null hypothesis that the means

of all the groups being compared are equal, and produces a statistic called F, which is equivalent to the t-statistic from a t-test.

For all statistical analysis, statistical significance was set at $p < 0.05$.

6. RESULTS: Characterization of autophagic process in experimental model of inflammatory and neuropathic pain

6.1 Peripheral nerve injury models

In this study we used three different models of neuropathic pain: SNL, SNI and CCI. All these models induced a rapid reduction in threshold of mechanical sensitivity on the ipsilateral side, but not on the contralateral side (Fig. 6.1 A, C, D). In the SNL model, mechanical allodynia starts 1 day after surgery and remains constant for at least 8 weeks (Fig. 6.1 A); no change was observed in the thermal sensitivity (Fig 6.1 B). In the SNI model, a reduction in threshold of mechanical sensitivity was observed starting 1 day after surgery, maximal sensitivity was reached 6 day later and kept constant for at least 14 days (Fig. 6.1 C). After CCI surgery, a robust mechanical allodynia developed starting from 1 day after surgery (Fig. 6.1 D).

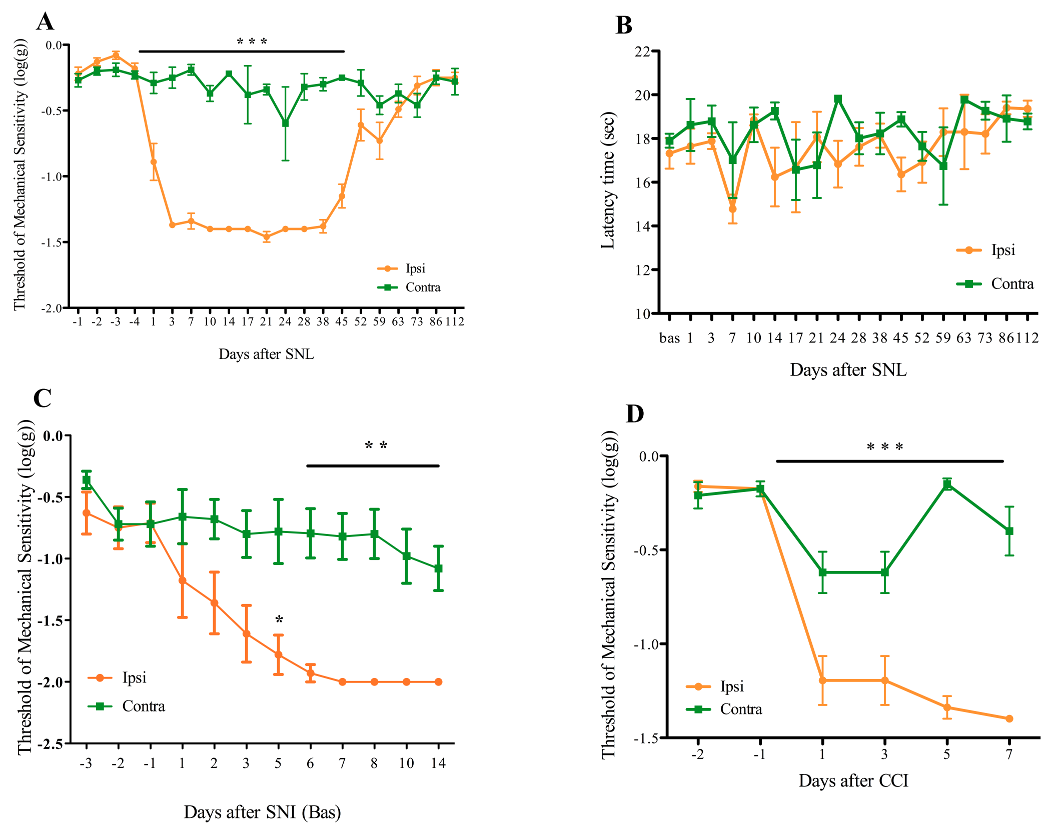


Fig. 6.1: Pain behaviour in different models of neuropathic pain. A) A severe and persistent hypersensitivity developed and maintained for at least 8 wks following SNL (n=6, ***P<0,001); **B)** SNL induced no changes in the thermal hypersensitivity over time after surgery (n=6); **C)** SNI induced a robust reduction of mechanical thresholds starting few days after injury (n=8, *P<0,05, **P<0,01); **D)** CCI surgery induced a reduction in threshold of mechanical sensitivity starting 1 day after injury (n=5, ***P<0,001). Data of mechanical sensitivity are expressed as means ± of 50% of pain threshold; data of thermal hyperalgesia are expressed as means of latency time and were normalized to the baseline of each animal.

6.2 Analysis of LC3 expression in the spinal cord

In our previous work, we showed an impairment of autophagy after SNL surgery (Berliocchi, 2011). Autophagy is one of the major intracellular degradation systems by which cytoplasmic materials are delivered to and degraded by lysosomes. LC3, a key protein in the autophagy pathway, is an ubiquitin-like protein that can be conjugated to phosphatidylethanolamine. The nonlipidated and lipidated forms are usually referred to as LC3-I and LC3-II, respectively. Monitoring LC3-II levels is considered a reliable method to estimate the rate of autophagosomes formation (Klionsky et al, 2012). In the present study, the expression of LC3 was examined by western blot analysis in the spinal dorsal horn 7 days after SNL and CCI, and 14 days following SNI (Fig. 6.2). An upregulation of LC3-I and increased levels of LC3-II in the ipsilateral (I) versus the contralateral (C) dorsal horn were observed in the SNL and SNI models as shown by western blot (Fig. 6.2 A) and confirmed by densitometric analysis (Fig. 6.2 B) (Fig. 6.5 A-B). On the contrary, no important variations in LC3-I expression or LC3-II levels were observed in the ipsilateral dorsal horn after CCI (Fig. 6.2 A and B) (Fig 6.5 C). In neuropathic pain model the calcium channel subunit $\alpha_2\delta$-1 is upregulated ipsilateral to the injury and its expression is confined to the lumbar portion of spinal cord (Bauer et al, 2009). Therefore, we used this protein as a reference for the specific lumbar segment and as a biochemical marker for a neuropathic pain state. In all the three models, $\alpha_2\delta$-1 upregulation was detected on the injured side (Fig. 6.2 A and B) thus confirming the correct sampling together with a state of pain and suggesting a different modulation of autophagy in the SNL and SNI versus the CCI model.

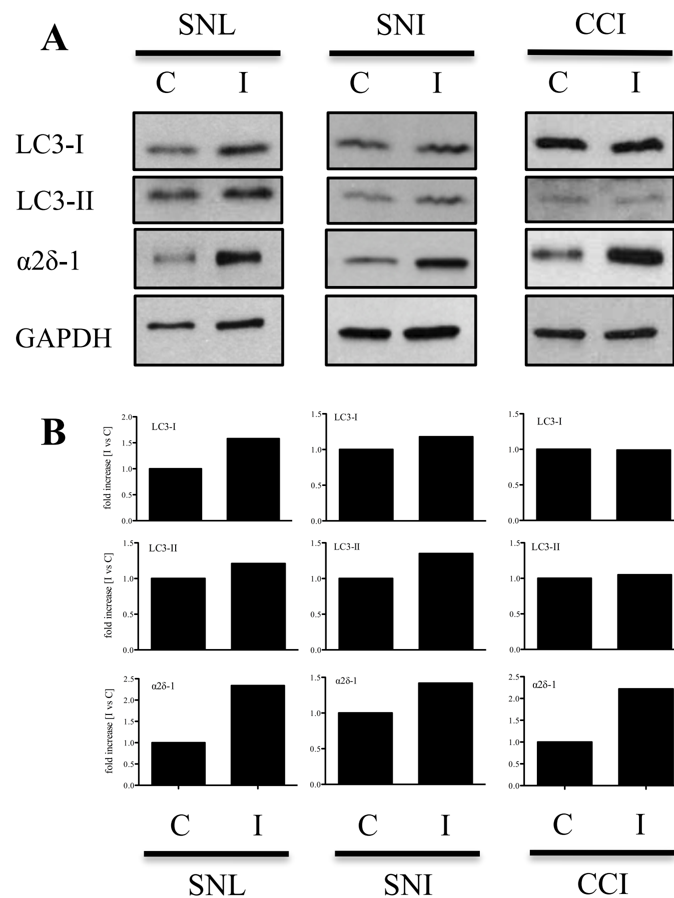


Fig. 6.2: Expression of LC3 in the spinal dorsal horn. (A) A representative western blot of LC3-I and LC3-II levels in the spinal dorsal horn following different models of peripheral nerve injury. LC3-I and LC3-II levels are modulated in the SNL and SNI but not in the CCI model (A and B). Upregulation of $\alpha_2\delta$-1was observed in the injured side of all models. Signals from each band were normalized towards the corresponding GADPH signal. **(B)** Variations of LC3 levels are confirmed by densitometric analysis

6.3 Beclin 1 and the induction of autophagy

Beclin 1 is a protein with a key role in the induction of autophagy as its expression is essential for the formation of autophagosome (Pattingre et al, 2008). To understand whether LC3-I increase and LC3-II formation was due to an upstream induction of autophagy, we analysed the expression of Beclin 1 in the three different models of neuropathic pain. Our results showed that in all the three models a trend towards an increase in Beclin 1 expression was detected on the injured side (Fig. 6.3 A and B) (Fig. 6.5 A-B-C) suggesting that after peripheral nerve injury there is only a mild induction of autophagy in the spinal cord ipsi lateral to the injury. In all the three models, $\alpha_2\delta$-1 upregulation was detected on the injured side (Fig. 6.3 A and B) thus confirming the correct sampling.

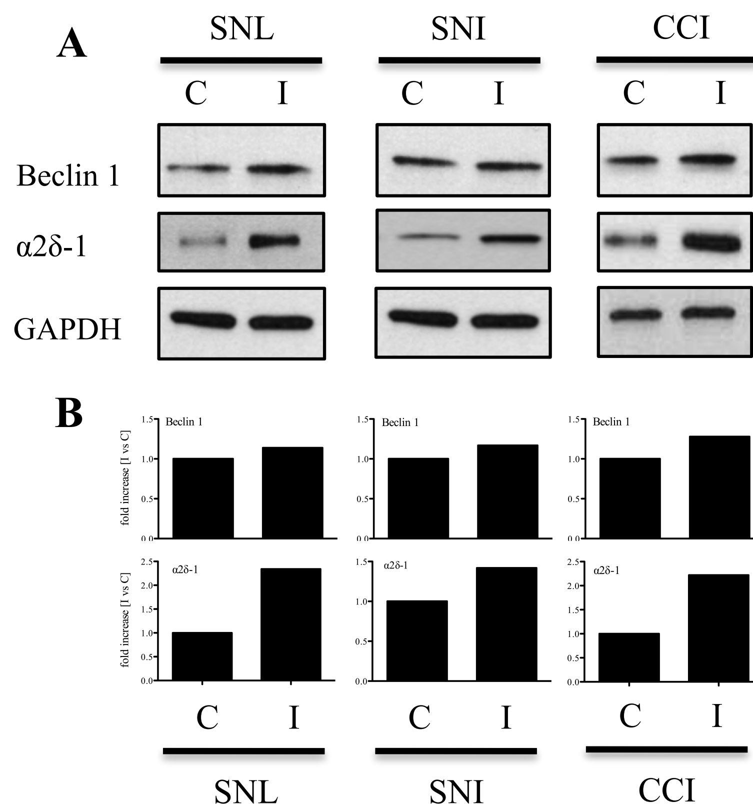


Fig. 6.3: Beclin 1 expression of in the spinal dorsal horn following peripheral nerve injury. (A) Representative western blots of Beclin 1 levels in the spinal dorsal horn in three different models of peripheral nerve injury. Upregulation of $\alpha_2\delta$-1was observed in the injured side of all models but only a trend towards an increase of Beclin 1 levels was detected (A and B). Signals from each band were normalized towards the corresponding GADPH signal. **(B)** Variations of Beclin 1 levels were analysed by densitometric analysis.

6.4 Analysis of p62 expression in the spinal cord

The protein p62, also know as sequestosome 1/SQSTM1, is a known substrate of selective autophagy (Mizushima and Komatsu, 2011). This protein interacts with LC3 on the isolation membrane, is incorporated into the autophagosome and, in normal conditions, degraded (Johansen and Lamark, 2011). Accumulation of p62 can therefore be a consequence of autophagy impairment (Komatsu et al, 2007).

To confirm our previous data (Berliocchi et al, 2011) and to test whether LC3-II formation was linked to autophagy impairment rather than induction, we evaluated p62 levels by Western Blot in the spinal dorsal horn of mice that underwent SNL, SNI and CCI. The expression of this protein was analysed in the L4-L5 portion of spinal cord ipsilateral (I) and contralateral (C) to the ligation, 7 days after SNL and CCI, and 14 days after SNI.

Samples from mice that underwent either SNL or SNI surgery showed upregulation of p62 levels ipsilateral to the ligation (Fig. 6.4 A) (Fig. 6.5 A-B). No difference in p62 levels was observed in the spinal dorsal horn of mice undergone CCI surgery (Fig. 6.4 A) (Fig. 6.5 C).

As for LC3 and Beclin 1, p62 expression was correlated with a2d-1 upregulation in the ipsilateral side of all three models. All the variations observed were confirmed by densitometric analysis of Western Blot results (Fig. 6.4 B).

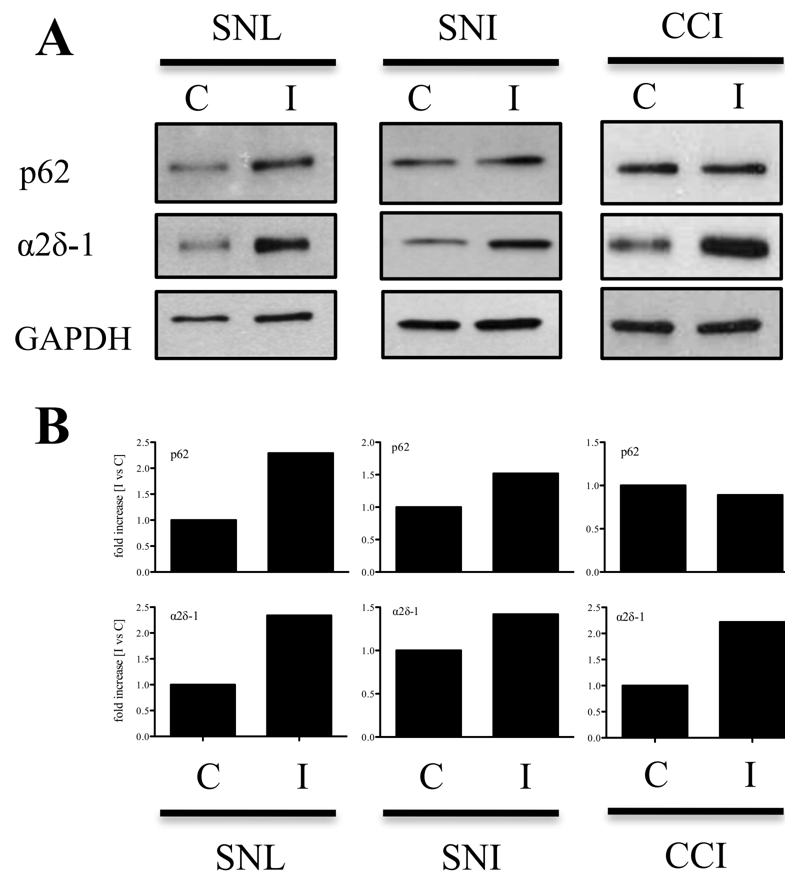


Fig. 6.4: p62 expression in the spinal cord following peripheral nerve injury. (A) p62 expression was analysed by Western Blot in the spinal dorsal horn ipsilateral (I) and contralateral (C) to the nerve injury in three different models of neuropathic pain. SNL and SNI surgery, but not CCI surgery, induced an upregulation of p62 in the spinal dorsal horn ipsilateral to the ligation. **(B)** The variation observed of the p62 levels, were confirmed by densitometric analysis. The signal of each band was normalized towards the corresponding GAPDH signal.

6.5 Statistical analysis

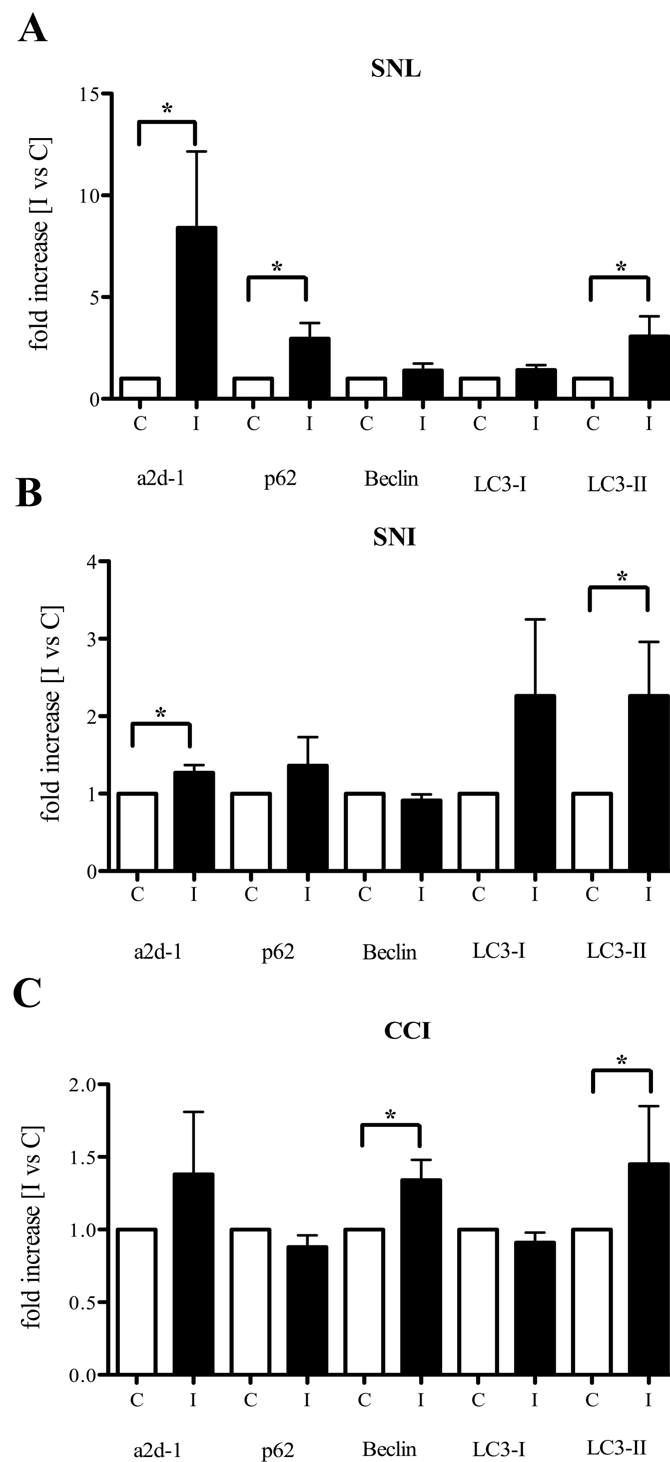


Fig. 6.5: Statistical Analysis. Quantitative analysis of Western blots by densitometry. Signals from each band were normalized towards the corresponding GAPDH signal. Value were expressed as mean ± SEM. * p < 0,05. SNL n=8; SNI and CCI n=5.

6.6 Expression and cellular localization of p62 in the spinal cord following peripheral nerve injury

In order to characterize the cellular distribution of the molecular changes observed by WB, double and triple immunofluorescent experiments with specific cellular markers were performed.

Since the currently available antibodies are not able to distinguish between the two forms of LC3 (LC3-I and LC3-II) we decided to focus own attention on the autophagy substrate p62. Immunohistochemistry showed that p62 was ubiquitously expressed in the spinal cord, with higher levels of expression in the superficial layer of the dorsal horn following SNI (Fig 6.6) and SNL (not shown). Results obtained by analysis of signal intensity showed a statistically significant upregulation of p62 expression on the ipsilateral side of injury (Fig. 4.6 B), thus confirming the western Blot results. The intensity of p62 immunoreactivity appeared stronger on the ipsilateral side than on the contralateral following SNI (Fig. 6.6 A) and SNL (not shown). A statistically significant increase on the ipsilateral versus contralateral side was confirmed by a quantitative analysis of intensity of fluorescence (Fig. 6.6 B), thus further supporting the western blot results.

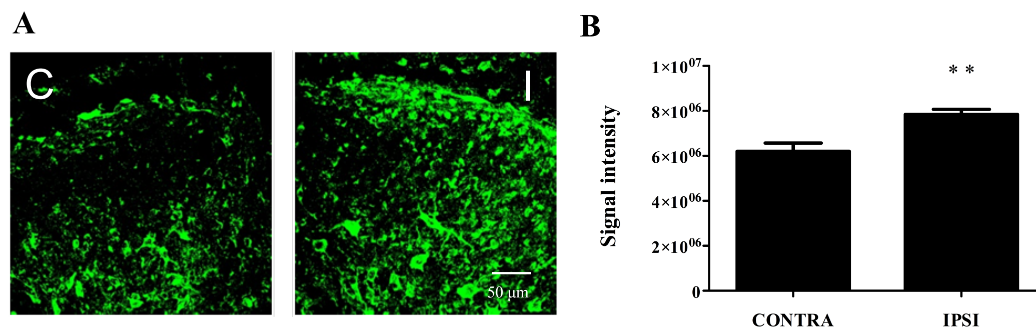
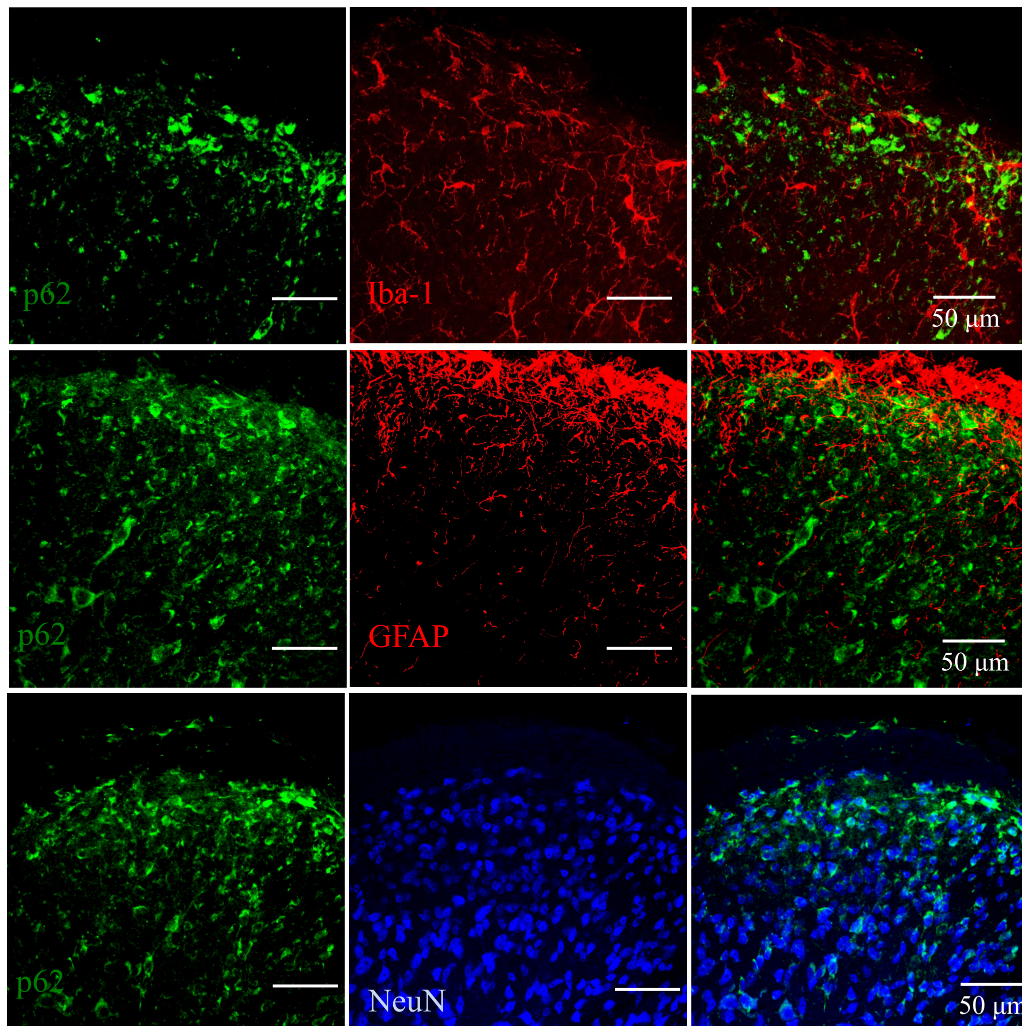


Fig. 6.6: Spinal increase in p62 immunoreactivity following peripheral nerve injury. (A) p62 positive cells were uniformly distributed in the spinal dorsal horn, especially in the superficial layer. However, the ipsilateral side showed higher p62 levels than the contralateral. **(B)** Analysis of p62 signal intensity was performed using Fiji software (n=7, P<0,01). Data are expressed as mean ± SEM.

To further characterize the cellular localization of p62, double and triple immunofluorescent stainings were performed using antibodies against p62, the neuronal marker NeuN, the astrocytic marker GFAP and the microglial marker Iba-1 (Fig. 6.7). Our results showed that p62 is mainly expressed in NeuN-positive cell bodies. However, strong p62-immunoreactivity can also be detected in the neuropile, suggesting that p62 might also be expressed in neuronal processes. No marked colocalization was observed with microglial and astocytic markers.



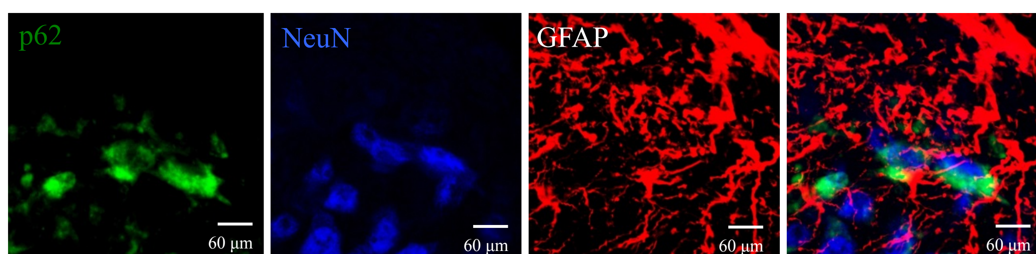


Fig. 6.7: Cellular localization of p62 in the spinal dorsal horn following SNI. Confocal images of mouse spinal dorsal horn sections. (A) co-staining of p62 (*green*) with NeuN (*blu*) GFAP (*red*); and Iba-1 (*red*) (B) particular of triple immunostaining of p62 localization within NeuN-positive cells. Confocal images show single focal planes.

6.7 Formalin-induced pain behaviour

Results from peripheral nerve injury suggested a role of autophagy in the pathophysiology on neuropathic pain. To understand whether this modulation of autophagic markers would be exclusively present in models of nerve injury and not in models on inflammation, we investigated the expression of autophagic markers in the formalin model.

Formalin injection induces a typical transient biphasic response in pain behaviour (Dubuisson and Dennis, 1977). The first phase seems to be caused by C-fibre activation due to the peripheral stimulus, while the second phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Tjølsen et al, 1992). Formalin injection in the plantar hindpaw induced the typical biphasic liking/biting behaviour monitored over 60’ (Fig. 6.8).

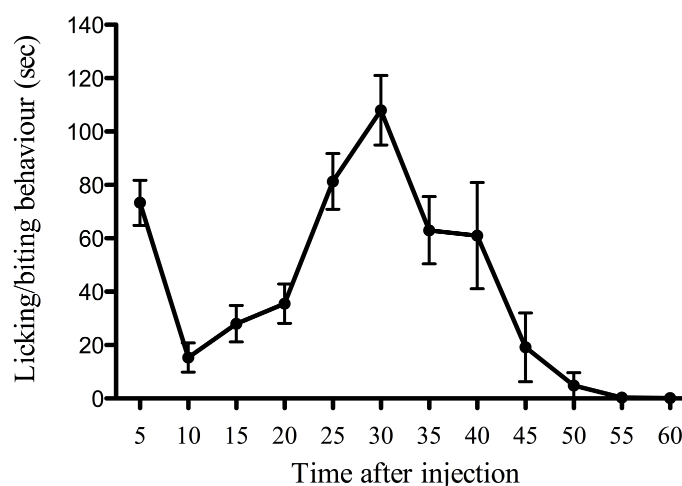


Fig. 6.8: Formalin-induced pain behaviour. Injection of formalin induced a biphasic behaviour, characterized by a first phase (5-10 min) and a second phase (15-60 min). Data are expressed as mean of liking/biting behaviour (n=9).

6.8 Analysis of autophagic markers after formalin injection

The spinal modulation of the autophagic process was also investigated in a model of inflammatory pain. The expression of the main autophagic markers was analysed at different time points in mice that received formalin injection in the hind paw. Classically, the formalin test is considered a model of acute inflammatory pain which produces two phases of nociceptive behaviour (Dubuisson and Dennis, 1977). However, formalin also produced long-term secondary thermal and mechanical hyperalgesia and allodynia in both paws (Fu et al, 2000; Fu et al, 2001; Fu et al, 2009; Vierck et al, 2008; Ambriz-Tututi et al, 2009; Wiertelak et al, 1994). The time points of 30 minutes, 2 hours and 4 days after formalin injection were chosen according to published data (Fu et al, 2001; Vierck et al, 2008; Ambriz-Tututi et al, 2009) and selecting the peak of the second phase (30 minutes), an early (2 hours) and a late (4 days) time point.

Thirty minutes after formalin injection, ipsilateral LC3-I levels were slightly increased and accompanied by a statistically significant formation of LC3-II and an increase of p62, compared to the contralateral side (Fig. 6.9). At 2 hours, ipsilateral LC3-I levels were slightly increased and accompanied by LC3-II and p62 levels comparable to the contralateral side (Fig. 6.9). However, p62 levels of both the ipsi and the contralateral side at 2 hours were comparable to the ipsilateral side at 30 minutes. At 4 days, ipsilateral LC3-I and LC3-II levels were comparable to the contralateral side whereas p62 levels in the ipsilateral were lower than in the side contralateral to injury. However, all three markers were present at much higher levels than at the previous time point. This seems to suggest a progressive activation of autophagy, indicated by LC3-II formation, LC3-I slight increase and p62 accumulation. However, these changes seem to increase over time and to extend to the contralateral side.

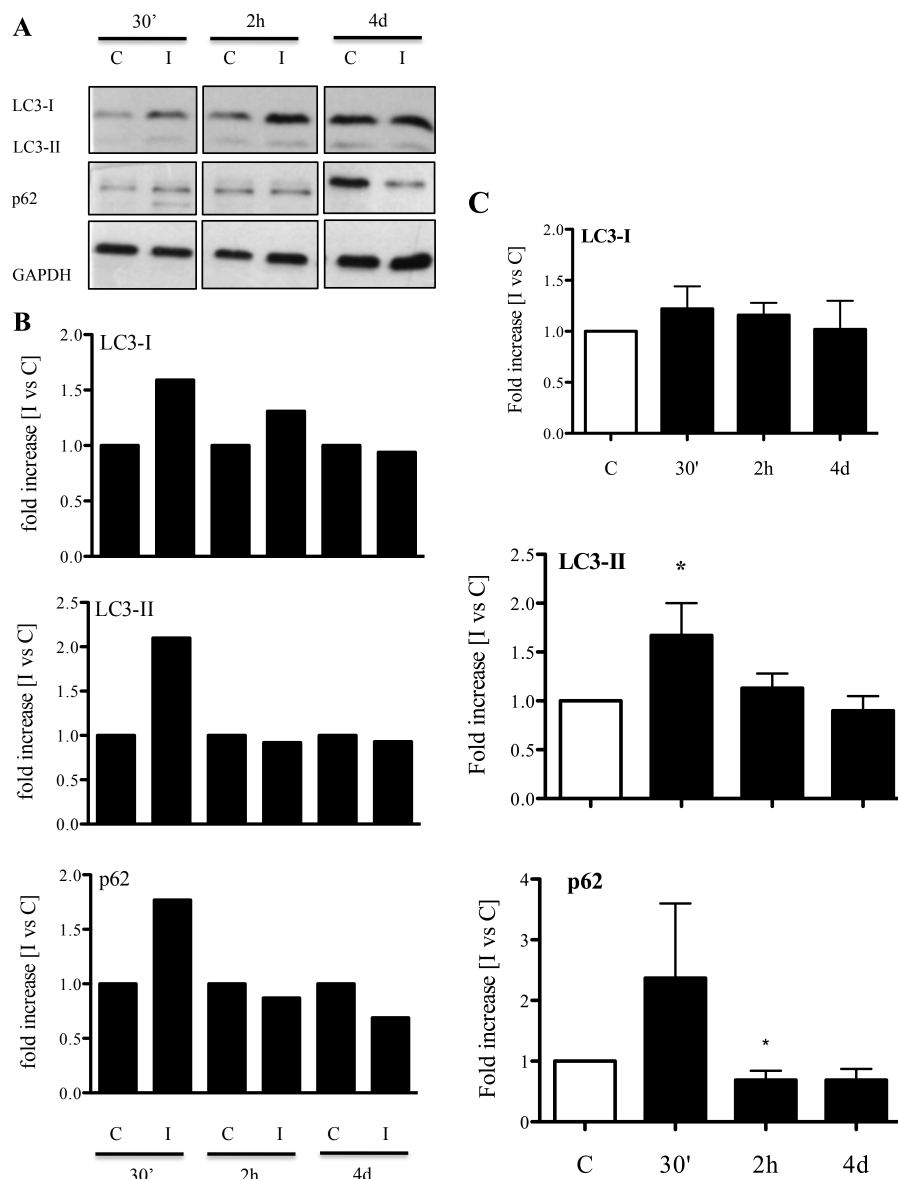


Fig. 6.9: Effect of Formalin on expression of autophagic markers. (A) Representative western blot of autophagic markers LC3 and p62 after formalin injection at different time points and **(B)** their densitometric analysis; **(C)** Statistical analysis on densitometry data. Compared to relative contralateral side, levels of LC3-II but not p62, were significantly enhanced 30 min after formalin injection. The density of each band was normalized to its respective GAPDH. Data are expressed as means ± SD (*P<0,05, n=6 for each group).

6.9 Spinal block of autophagy results in pain behaviour

In order to verify whether a localized block of autophagy at spinal cord level, can be relevant and have consequence for pain processing, we used the anti-malaria drug chloroquine (CQ) as a pharmacological tool to block autophagy. Together with bafilomycin A_1 and NH_4Cl, CQ is one of the main chemicals used to inhibit autophagy. Like NH_4Cl, CQ neutralises the lisosomal pH, and by inhibiting endogenous protein degradation causes the accumulation of sequestrated material in either autophagosomes or autolysosomes. Moreover, CQ may possibly block autophagosomes fusion with lysosomes (Klionsky et al, 2012). Chloroquine (100 μM) was administered to naïve mice by a daily intrathecal injection for 3 consecutive days. Behavioural test were conducted daily 2h after injection and samples from lumbar portion of spinal cord were then analysed by Western blot according to the experimental scheme in Fig. 6.10.

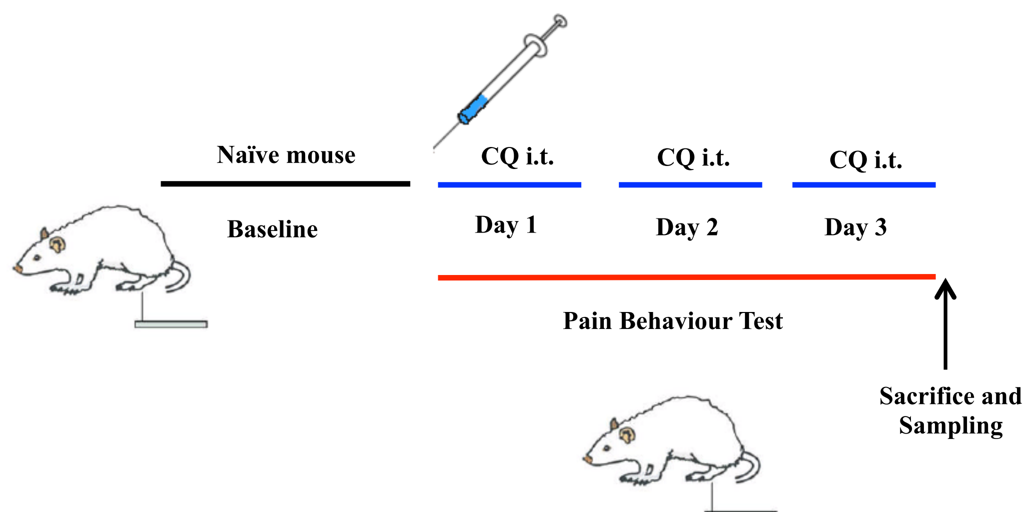


Fig. 6.10: Experimental protocol for Chloroquine treatment

As shown in figure 6.11, chloroquine treatment induced LC3-II formation and p62 accumulation in treated mice, when compared to vehicle-injected mice, thus confirming the drug action on the autophagic machinery. Moreover, the behavioural test (Von Frey's) carried parallely to the treatment showed a significant reduction of threshold in mechanical sensitivity starting from day 2 in chloroquine-injected mice in comparison to vehicle-injected mice (Fig. 6.12). These results suggest that spinal autophagy may be relevant for pain processing.

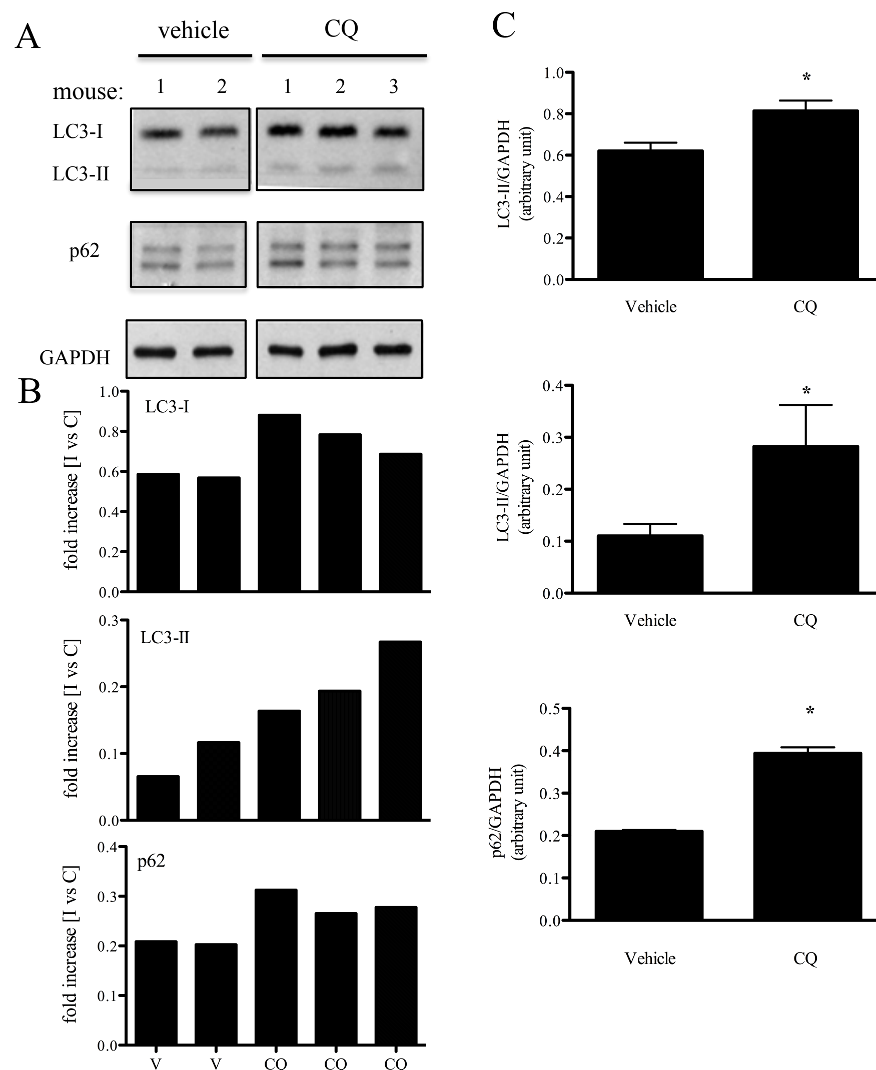


Fig. 6.11: Effect of i.t. Chloroquine on LC3-II and p62 accumulation in the spinal dorsal horn. Representative western Blot of LC3 and p62 protein levels **(A)** and relative quantitative analysis **(B)** after chloroquine treatment; **(C)** Statistical analysis of LC3 and p62 expression. Compared with the vehicle-injected mice, levels of LC3-I, LC3-II and p62 were significantly enhanced in the chloroquine-injected group. The band densities were normalized to GAPDH. Values are means ± SD (*P<0,05, vehicle n=3, CQ n=6).

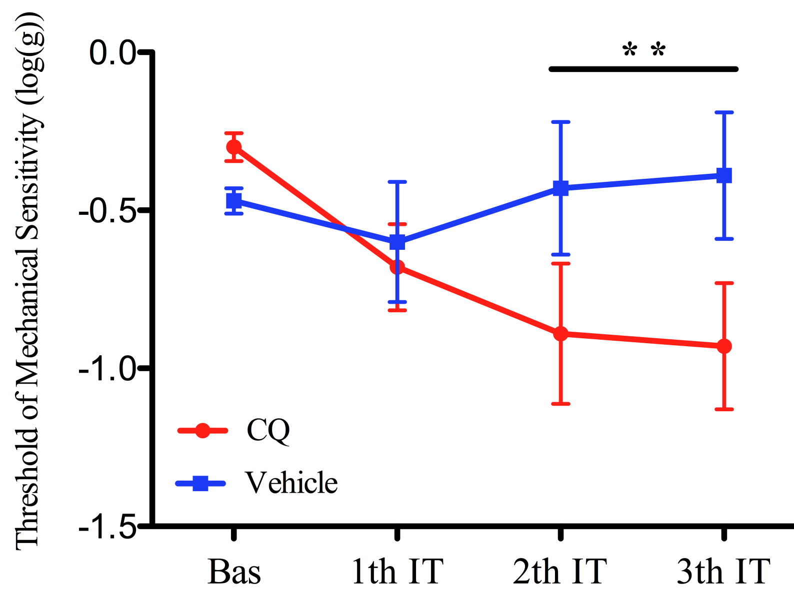


Fig. 6.12: Effect of Cloroquine (CQ) on the mechanical sensitivity. Naïve mice were injected with CQ (100 µM) daily for three days. Localised spinal block of autophagy significantly reduced the threshold of mechanical sensitivity, in comparison to vehicle-treated mice. Data are expressed as means ± SEM of 50% of the threshold of mechanical sensitivity (vehicle n=3, CQ n=6, **P<0,01).

7. RESULTS: Study of the role of the gene FKBP5 in the modulation of pain states; involvement of epigenetic mechanisms

7.1 Role of FKBP5 in models of short-lasting inflammation

First we investigated to role of FKBP5 gene in models of short-lasting inflammation. Therefore, we selected models of local tissue inflammation induced by injection of agents such as IL-6 and formalin into the mice hind paw, known to induce a short-lasting (maximum few hours) state of inflammation and pain behaviour (Dubuisson and Dennis, 1977; Ferreira et al, 1979).

a. There is no difference in nociceptive behaviour between WT and FKBP5 -/- mice following formalin injection

Injection of formalin into the hind paw produced localized short-lasting inflammation. The pro-nociceptive effect of formalin, recorded as liking/biting of the injected paw, is biphasic (Fig. 7.1A). Formalin-induced nociceptive behaviour was the same in wild type (WT) and FKBP5 -/- mice (Fig. 7.1A).

The protein Fos is expressed in spinal dorsal horn neurons involved in pain processing following noxious stimuli (Hunt et al, 1987), including formalin injection (Presley et al, 1990). In the present study, injection of 5µl formalin resulted in the appearance of c-Fos labelled nuclei mainly in the superficial laminae (Fig 7.1B). There was no difference in the number of c-Fos positive cells between wild type and FKBP5 -/- mice (Fig. 7.1C).

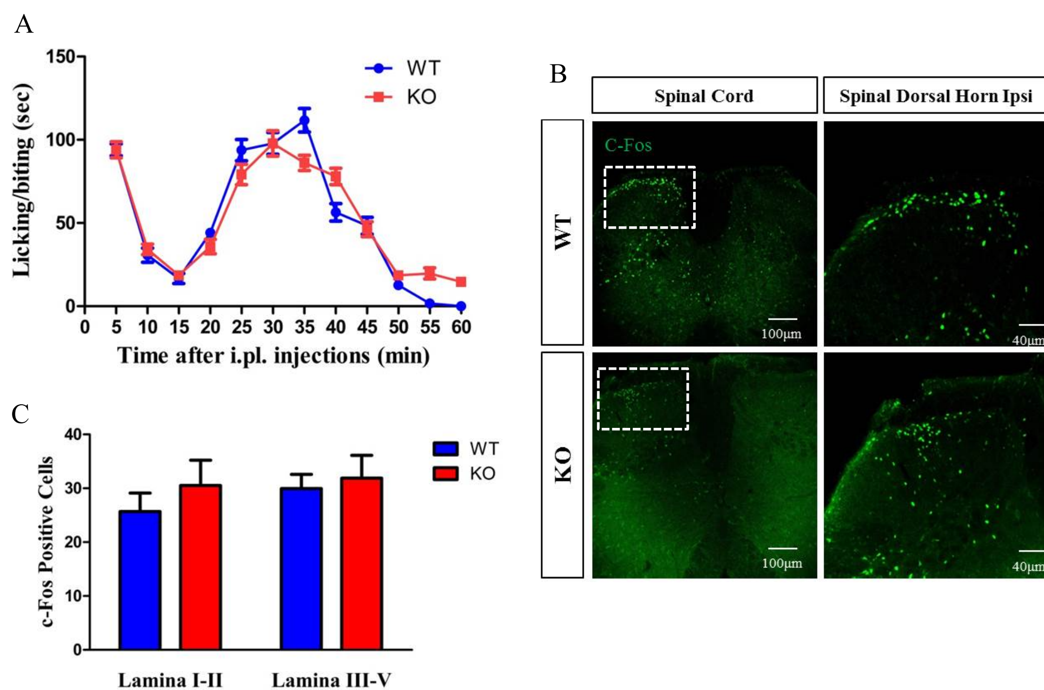


Fig. 7.1: There is no difference in nociceptive behaviour between WT and FKBP5 -/- mice after formalin injection. (A) No significant difference in nociceptive behaviour was observed between wild type and FKBP5 -/- mice after formalin injection (n=7 for each group). Data are means ± SEM of time of licking/biting behaviour; **(B)** Immunohistochemistry for c-Fos in the dorsal horn of formalin-injected mice 2h after injection; **(C)** Number of c-Fos positive cells in the superficial dorsal horn (Laminae I-II) and in laminae III-V. Data show group mean ± SEM.

b. There is no difference in pain behaviour between WT and FKBP5 -/- mice following PGE_2 injection

Injection of PGE_2 into the hind paw results in a transient reduction in threshold of mechanical sensitivity that resolves in one week (Aley et al, 2000). This effect was observed only in the injected paw, without any change in the contralateral side (Fig. 7.2A). FKBP5 -/- and wild type mice displayed the same nociceptive behaviour following PGE_2 injection (Fig. 7.2B).

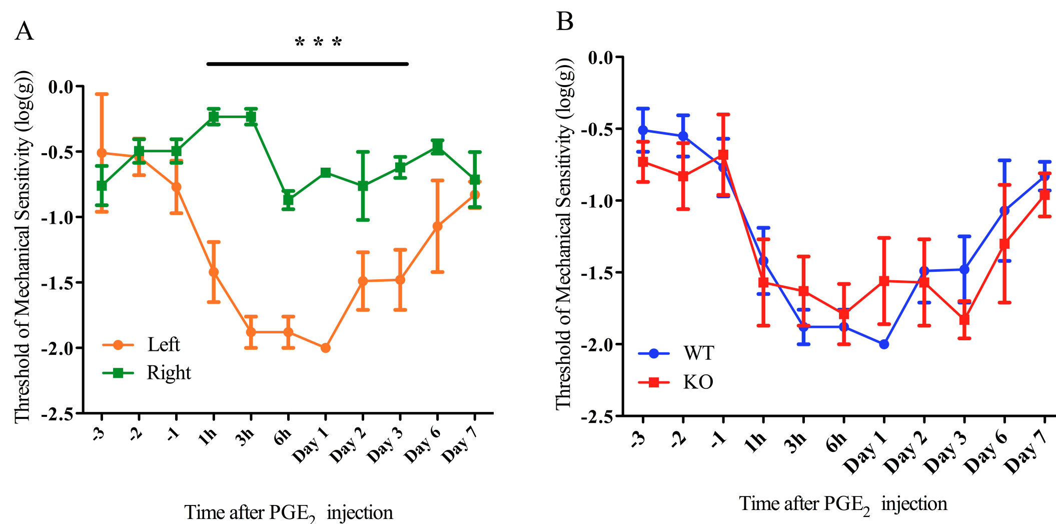


Fig. 7.2: There is no difference in mechanical threshold between WT an FKBP5 -/- mice following PGE_2 injection. (A) Injection of PGE2 in the plantar surface of the paw resulted in a reduced threshold of mechanical sensitivity in the injected paw. No effect was observed on the contralateral side (N=4, ***P<0,001); (B) No difference in mechanical threshold was observed between wild type and FKBP5 -/- mice after PGE2 injection (n=4).

7.2 Role of FKBP5 in models of long-lasting inflammation

Local, long-lasting inflammation was induced by injecting Complete Freud Adjuvant (CFA) either in the ankle joint or in the hind paw of mice. In these models nociceptive behaviour lasts a minimum of 7 days but does not involve irreversible damage to the body.

a. Behavioural validation of CFA induced joint inflammation

The injection of CFA produces persistent pain and hyperalgesia (Iadarola et al, 1988). In agreement with previous observation, CFA injected in the ankle joint of mice induced a rapid reduction in mechanical threshold (Fig. 7.3), that reached its maximum 24h after injection and remained constant for at least 7 days (Fig. 7.3) ($F_{1,13}$=333.304, p<0,001, n=8). No reduction in mechanical threshold was observed in the contralateral (right) side at any time.

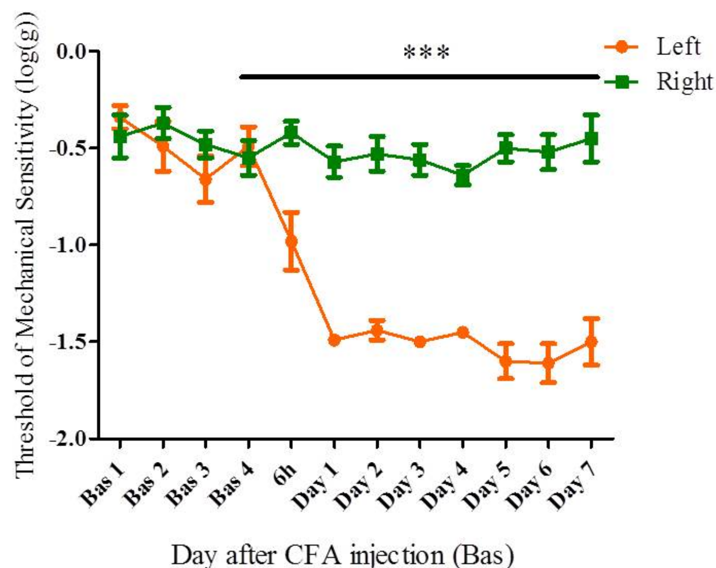


Fig. 7.3: CFA injection into the ankle joint induces a fast decline of mechanical threshold. A significant decrease in withdrawal threshold to mechanical stimulation was observed from 6h after CFA injection in the ipsilateral side. Maximal sensitivity was reached 1 day after injection and maintained constant for at least 7 days. No reduction in pain threshold was observed in the contralateral side (n=8, ***P<0,001). Data are expressed as means ± of 50% of the pain threshold.

b. FKBP5 -/- mice are less sensitive than WT to mechanical stimulation following CFA injection in the ankle joint

To evaluate the consequence of FKBP5 deletion on changes in mechanical sensitivity, FKBP5 -/- and wild type mice were injected with CFA in the ankle joint and tested daily with von Frey filaments to monitor possible changes in mechanical threshold. In wild type mice an important reduction of withdrawal threshold to mechanical stimulation was observed starting from 6h after injection. Maximal sensitivity was reached 1 day after injection and maintained for at least 7 days. Only a very small reduction in threshold of mechanical sensitivity was observed, instead, in FKBP5 -/- mice. ($F_{(1,13)}$ = 26 627, p<0,001; WT n=7, KO n=8) that never reached wild type levels (Fig. 7.4).

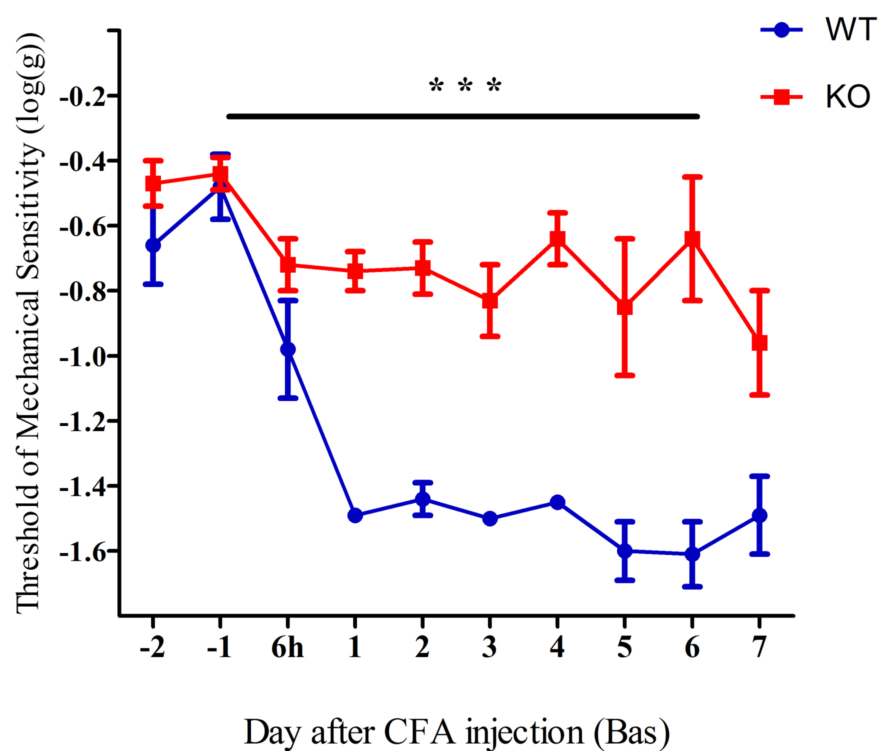


Fig. 7.4: FKBP5 -/- mice are less sensitive than WT to mechanical stimulation following CFA injection. The withdrawal Threshold to mechanical stimulation was drastically reduced in WT mice after CFA injection in the ankle joint, whereas only a minor reduction was observed in FKBP5 -/- mice. (p<0,001, WT n=7, KO n=8). Data are expressed as means ± of 50% of the pain thresholds.

c. FKBP5 -/- mice are less sensitive than WT to mechanical stimulation following CFA intraplantar injection

To be able to assess primary hyperalgesia (Meyer et al, 2006), namely the first nociceptive response free of secondary sovra-spinal amplification process, in a second set of experiment the inflammatory drug CFA was injected in the mice hind paw.
No difference was observed in thermal hyperalgesia after intraplantar CFA injection in the hind paw between FKBP5 -/- and wild type mice (Fig. 7.5).

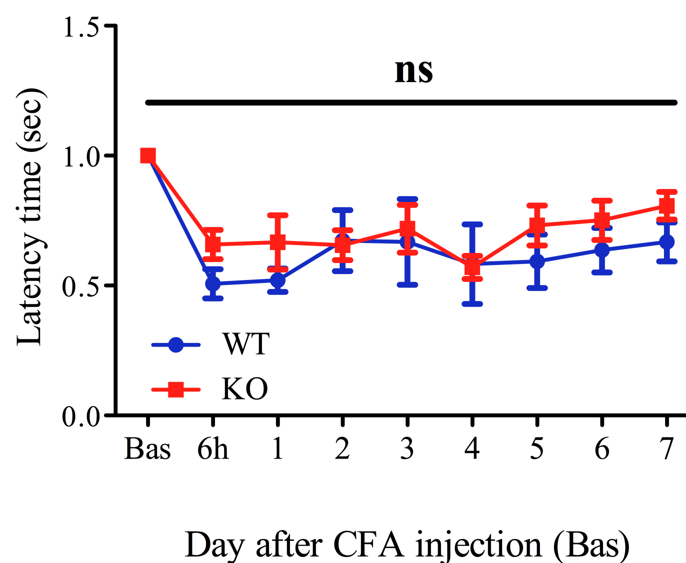


Fig. 7.5 : There is no difference in thermal threshold between FKBP5 -/- and wild type mice after intraplantar CFA injection. Latency time to withdrawal to thermal stimulation (n=8 for each group).

This model has been previously shown to induce a robust reduction of mechanical threshold as described elsewhere (Stein et al, 1988). This was confirmed by a strong reduction in threshold of mechanical sensitivity in wild type mice starting from 6h after injection (Fig. 7.6A) (n=8, ***p<0,001, $F_{1,14}$= 21.389). On the contrary, FKBP5 -/- mice showed a significant decrease in mechanical threshold that paralleled, but never reached wildtype levels (Fig. 7.6A) (n=8, **p<0,01). This decrease was only 77% of that of wild type mice (when compared to baseline) (Fig. 7.6A) (n=8, **p<0,01).

Since intraplantar CFA injection induces edema and inflammation in the injected paw, we verified that the difference observed in mechanical allodynia was not linked to a different degree of inflammation by measuring the thickness of the injected paw. In both genotype, the paw size was significantly increased in comparison to control mice, but no difference was observed between WT and FKBP5 -/- mice (Fig. 7.6B) (n=8 for each group, *** p<0,001, $F_{1,2}$= 70.765).

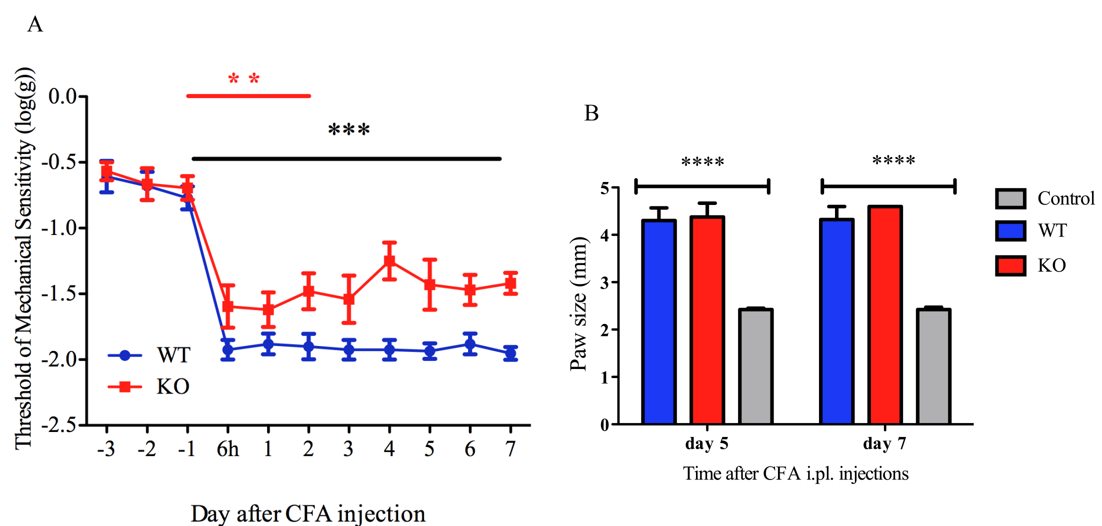


Fig. 7.6: FKBP5 -/- mice are less sensitive than WT to mechanical stimulation after intraplantar CFA injection. (A) Threshold of mechanical sensitivity in FKBP5 -/- and WT mice after intraplantar CFA injection and **(B)** Measure of paw size. Data are expressed as mean ± SEM. (n=8 for each group, ** p<0,01, *** p<0,001).

7.3 Role of FKBP5 in a neuropathic pain model

We investigated to role of FKBP5 gene in a model of long-lasting neuropathic pain. Therefore, we selected model of Spared Nerve Injury, described by Decosterd and Woolf (2010). This model involves damage to a nerve and it is useful to mimic damage to the peripheral nervous system seen in clinical settings.

a. **FKBP5 -/- mice show lower mechanical hypersensitivity after spared nerve injury.**

To evaluate the effect of FKBP5 deletion on a neuropathic pain model, wild type and FKBP5 -/- mice were subjected to SNI surgery. This model of neuropathic pain is characterized by a persistent mechanical allodynia that starts few days after surgery (Decosterd and Woolf, 2000). A statistically significant reduction of pain threshold was observed in WT mice. However, FKBP5 -/- mice showed only an initial reduction of pain threshold (*p<0,05), that stabilised and never reached the decrease in sensitivity seen in WT mice (Fig 7.7A). ($F_{(1,13)}$ =31 559, p<0,001; WT n=7, KO n=8).

A typical feature of the SNI model is the development of motor deficit after surgery (Urban et al, 2011). To test whether the differences in mechanical sensitivity between FKBP5 -/- and wild type mice could be linked to differences in motor deficit, we tested wild type and FKBP5 -/- mice that underwent SNI surgery versus naïve mice on accelerating Rotarod (Ugo Basile, Italy). In agreement with the behavioural data, KO mice stayed on the apparatus for a significantly longer time than WT mice and comparable to naïve mice (Fig. 7.7B) (*t. test* * p<0,05, Naïve n=4, WT n=9, KO n=8).

Thermal responsiveness to cold stimuli is increased in the ipsilateral side after SNI surgery (Decosterd and Woolf, 2000), and our results showed no difference between WT and KO mice in the acetone test (Fig. 7.7C), but these data need to be confirmed and extended (n=4 for each group).

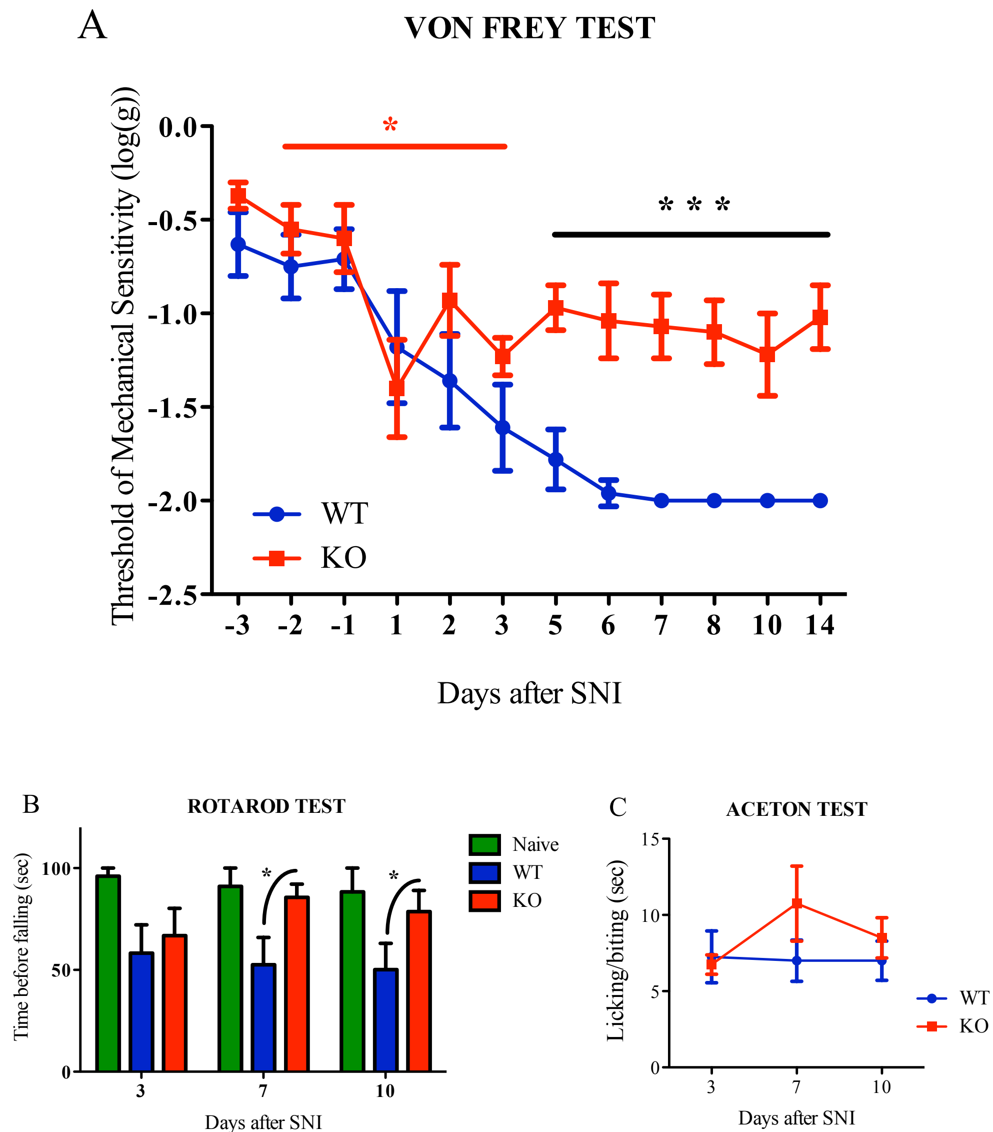


Fig. 7.7: Effect of FKBP5 deletion on pain-like behaviour after SNI. (A) Evaluation of withdrawal threshold to mechanical stimulation using the Von Frey's Test (WT n=7, KO n=8); **(B)** Latency to fall from Rotarod apparatus (Naïve n=4, WT n=9, KO n=8); **(C)** Withdrawal duration to cold using the aceton test (n=4 for each group). Data are expressed as mean ± SEM. (* p<0,05, *** p<0,001).

7.4 Analysis of glial activation after SNI surgery

In response to most types of peripheral nerve injury, an increased glial activation has been observed in the spinal cord ipsilateral to the surgery. These changes often contribute to the onset and maintenance of pain-like behaviour (Watkins et al, 2001). Furthermore, recently Golovatscka and colleagues suggested that stress could activate cytokines and immune response in dorsal horn and DRGs without any noxious stimulation (Golovatscka et al, 2012). To determine whether the difference in pain behaviour following SNI observed in WT and KO mice could be linked to a different activation of glial cells, we investigated dorsal horn immunoreactivity for the glial marker GFAP and for the microglial marker Iba-1. First, our results confirmed that the glial activation occurs after noxious stimulation: 14 days after SNI GFAP upregulation and a statistically significant Iba-1 upregulation were observed in the dorsal horn ipsi lateral to injury in WT mice (Fig. 7.8 A-B). Analogous changes in glial reactivity were observed in FKBP5 -/- mice and a statistical significant upregulation was also observed for Iba1 ipsi lateral to the surgery (Fig. 7.8 C-D).

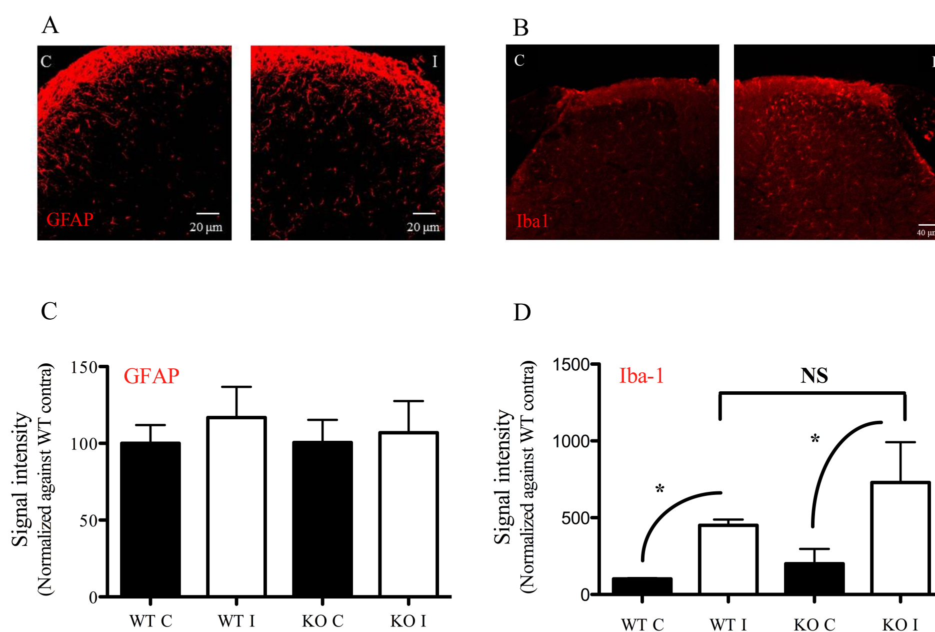


Fig. 7.8: SNI surgery induces the same increase in glial activation in FKBP5 -/- and wild type mice. SNI surgery induced upregulation of GFAP and Iba1 positive cells ipsilateral to the injury in wild type mice (A and B); signal intensity analysis showed no differences between WT and KO in the glial activation after SNI surgery (C and D). (WT n=7, KO n=8).

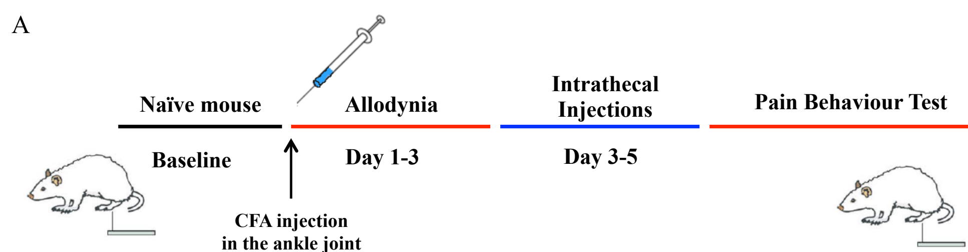
7.5 Localized silencing of FKBP5 at lumbar level reduce mechanical sensitivity

Since the FKBP5 -/- mice are global knockouts, we decided to examine a more specific role of FKBP5 on pain processing at the spinal dorsal horn. For this, we silenced the gene only in the lumbar area of the spinal cord using antisense technology. The siRNA was injected intrathecally in WT mice, deleting the gene in the cord but allowing its expression in the rest of the body. FKBP5 silencing was performed in conjunction with CFA injection in the ankle joint, a model that has been previously shown to induce FKBP5 gene expression (Géranton et al, 2007). Two different experimental schemes were followed.

a. FKBP5 silencing during a full pain state

In this set of experiments, the antisense was injected 3 days after CFA injection, when the pain state was already completely developed (Fig. 7.9A). Mice that received the antisense displayed a rapid recovery starting from the second intrathecal injection and lasting until day 10 after CFA injections (Fig. 7.9B) (n=8 for each group, $F_{1,13}$= 41.995, *** $p<0,001$). This suggests that FKBP5 is involved in pain processing in the dorsal horn and in particular in the maintenance phase of chronic pain.

A



B

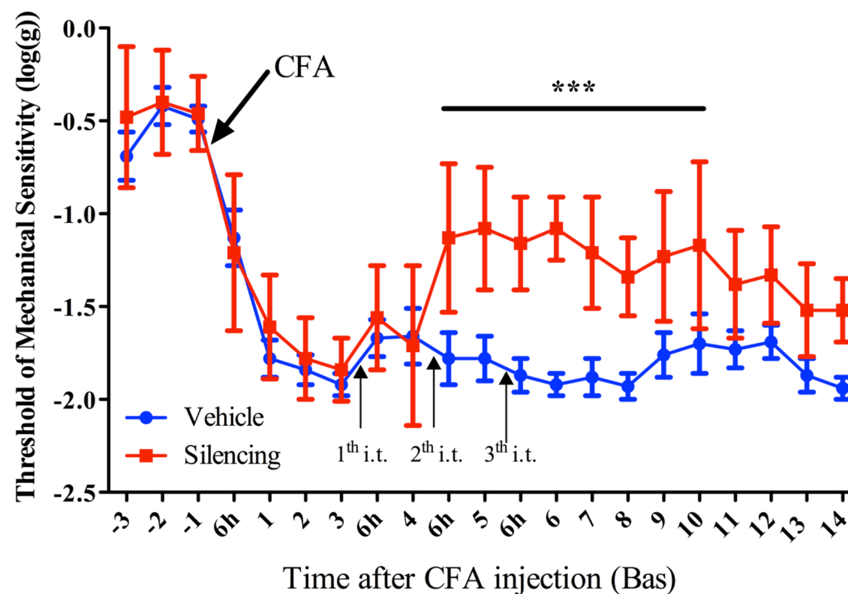


Fig. 7.9: Local silencing of FKBP5 in the lumbar cord following CFA reduces the decrease in mechanical threshold seen after CFA injection in the ankle joint. (A) Experimental protocol used in (B). The antisense was injected intratecally for 3 consecutive days, starting from day 3 after CFA injection in the ankle joint. The mice were subjected to behavioural test daily and 6h after intrathecal injection; **(B)** Antisense-injected mice are less sensitive than vehicle-injected mice. Data shown mean ± SEM of 50% of mechanical threshold. (n=8 for each group, *** p<0,001).

b. Silencing FKBP5 before the development of a full pain state

To understand the involvement of FKBP5 in the induction phase of chronic pain, we decided to silence the gene before the pain development. The antisense was injected daily for 2 consecutive days intratecally in naïve mice and Von Frey's test performed (Fig. 7.10A). No difference in pain behaviour was observed between vehicle- and antisense- injected mice (Fig. 7.10B), and also no difference was observed in the contralateral side during the whole study (data not shown), indicating that the antisense did not have any effect *per se*. On the third day, mice received both an injection of siRNA and CFA in the ankle joint. Following CFA injection, antisense-

injected mice displayed a lower degree of mechanical hypersensitivity in comparison to vehicle-injected mice (Fig. 7.10C) (n=8 for each group, $F_{1,14}$= 13.396, ** p<0,01). These results confirmed the involvment of FKBP5 in pain processing in the spinal cord and suggested the involvment of this gene also in the induction phase of chronic pain.

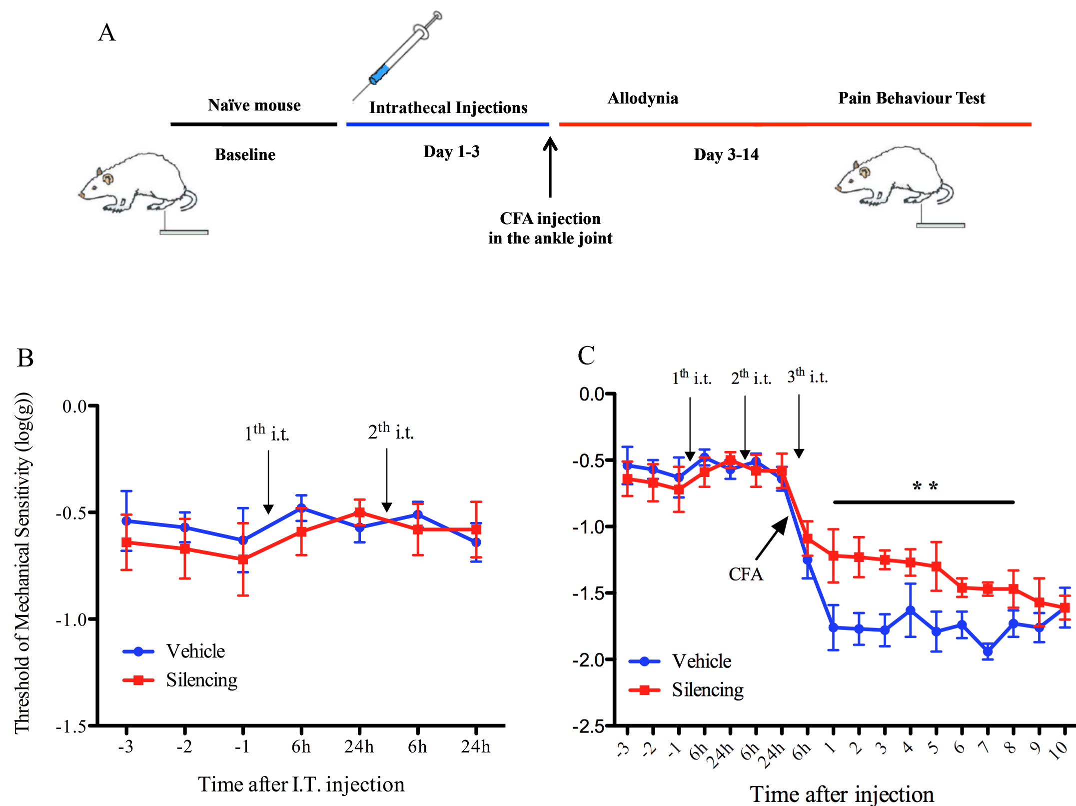


Fig. 7.10: Local silencing of FKBP5 in the lumbar cord prior to CFA injection reduces the decrease in mechanical threshold seen after CFA injection in the ankle joint. (A) Experimental protocol used in (C). Antisense was injected intrathecally daily for 2 days before CFA injection and the mice were subjected to behavioural test; **(B)** FKBP5 Silencing had no effect on the baseline threshold of mechanical sensitivity; **(C)** Antisense injected mice are less sensitive after CFA injection if compared with vehicle injected mice. Data are expressed as mean ± SEM of 50% of mechanical threshold. (n=8 for each group, ** p<0,001)

7.6 Setup of protocol of hyperalgesic *priming*

There are scientific evidence for a role of FKBP5 in priming stress responses (Klengel et al, 2013). To test the hypothesis that FKBP5 could also primes pain response, we set up a protocol of hyperalgesic priming, as described by Levine's group (Reichling and Levine, 2009).

Intraplantar administration of IL-6 (25 µl) causes short-term localized signs of inflammation (swelling and erythema) and a decrease in the mechanical paw withdrawal threshold in the injected paw measured 1h and 3h after injection. Both the visible signs of inflammation and the decrease in paw withdrawal threshold resolved within 72h after the IL-6 injection (Fig. 7.11 A). Thus, 7 days after IL-6 injection, there was no difference in baseline paw withdrawal threshold between mice injected with IL-6 (Primed mice) and control injected with vehicle. However, intraplantar injection of the inflammatory mediator PGE_2 at the same site where IL-6 had been injected 7days earlier resulted in a prolonged mechanical hyperalgesia that persisted for at least 3 weeks after IL-6 injection. On the contrary, in control mice that received the vehicle, and in naïve mice, PGE2 produced a transient hyperalgesia lasting only 6h (Fig. 7.11 B).

The model represents an essential experimental tool and will be used for further work on the role of FKBP5 in different pain settings.

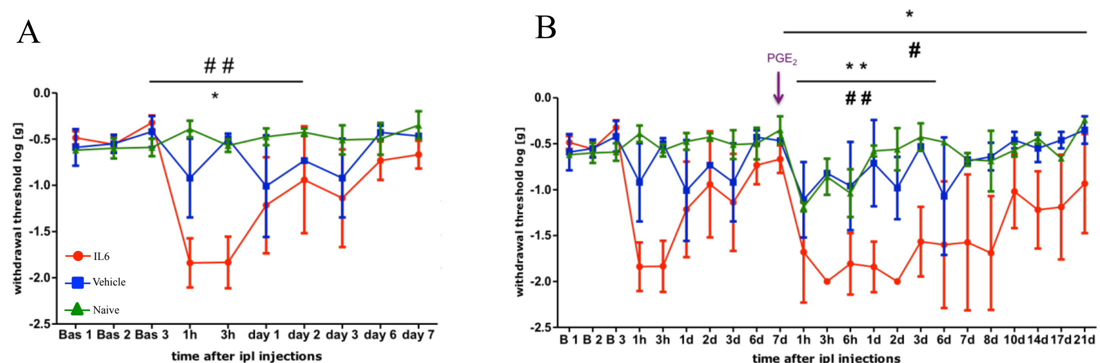


Fig. 7.11: Persistent nociceptive sensitization initiated by IL-6 injection. (A) In mice that received IL-6 intraplantary withdrawal threshold to mechanical stimulation (von Frey) decreased significantly from 1h after injection. No reduction in pain threshold was observed in mice that received only vehicle or in naïve mice; **(B)** PGE2 injected into the hindpaw on day 7 after IL6 injection, produced a strong and persistent sensitization that was statistically significant from 1h until 3 days after injection, and is maintained for at least 3 weeks (*p<0,05; **p<0,01; Pilot Experiment n=3; # naïve vs IL6, * Veh vs IL6). Data show mean ± SEM.

7.7 Characterization of the enzymes involved in the regulation of DNA methylation: DNMT1 and TET1

a. Expression of DNMT1 in different models of chronic pain

An increase in DNMT1 has been recently described in the spinal cord 7 days following CFA injection in the ankle joint (Tochiki et al, 2012). However, DNMT1 expression was only investigated at mRNA level using RT-qPCR.

Here, DNMT1 expression was further investigated at protein level and its cellular expression characterized in the same CFA model, but also extended to a neuropathic pain model like SNI.

In naïve animals DNMT1 is mainly expressed in neuron than in astrocytes in the superficial dorsal horn

The expression of DNMT1 in the superficial dorsal horn of naïve animals is significantly greater in neurons than in astrocytes (*** p< 0,001) as shown in figure 7.12.

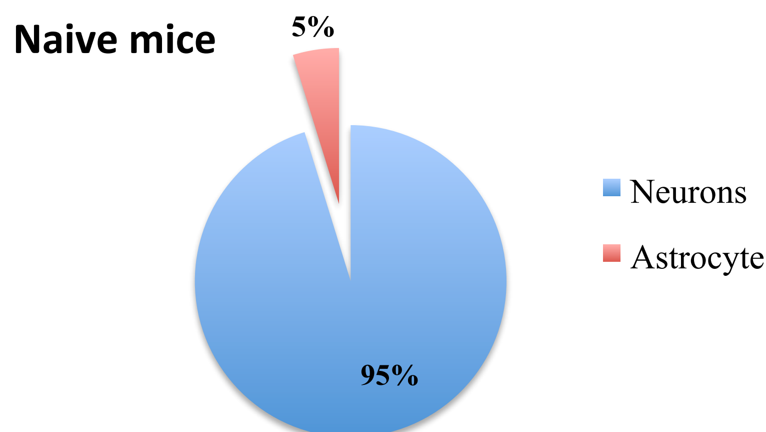


Fig. 7.12: Expression of DNMT1 is significantly greater in Neurons than Astrocytes in the Superficial Dorsal of Naive Animals. The figure shows that DNMT1 expression

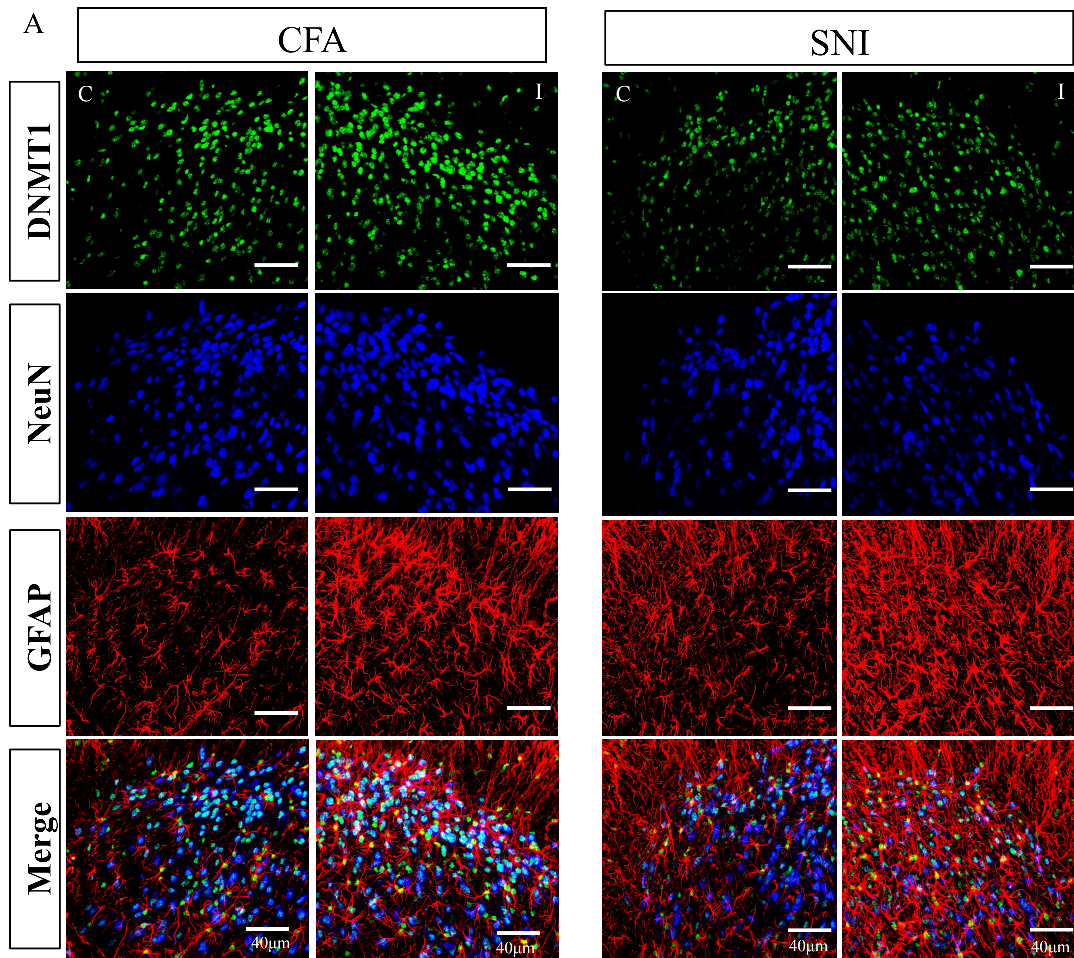
Seven days after the pain stimulus, the neuronal and glial expression of DNMT1 was investigated in the superficial dorsal horn of animals that underwent either SNI surgery or received a CFA injection in the ankle joint and their relative sham animal. Using a triple staining protocol, the expression of DNMT1 was analysed in neurons and astrocytes, using NeuN and GFAP as a marker (Fig. 7.13 A).

CFA injection in the ankle joint causes a significant change in DNMT1 expression in neurons and astrocytes

Analysis of DNMT1 signal intensity in neurons and astrocytes, performed with Fiji software, showed a significant upregulation in CFA-injected animals compared with sham animals in both cell types (Fig. 7.13 E) (n=4 for each group, ** $p<0,01$).

SNI surgery causes a significant increase in DNMT1 expression 7 days after injury

Statistical analysis of tissue sections showed that SNI surgery causes a significant increase in DNMT1 expression in astrocytes in the superficial dorsal horn (* $p<0,05$) suggesting that this enzyme could be more important in astrocytes than in neurons in this model of chronic pain (Fig. 7.13 D).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CFA
SNI
C
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C
I
DNMT1
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GFAP
Merge
40µm
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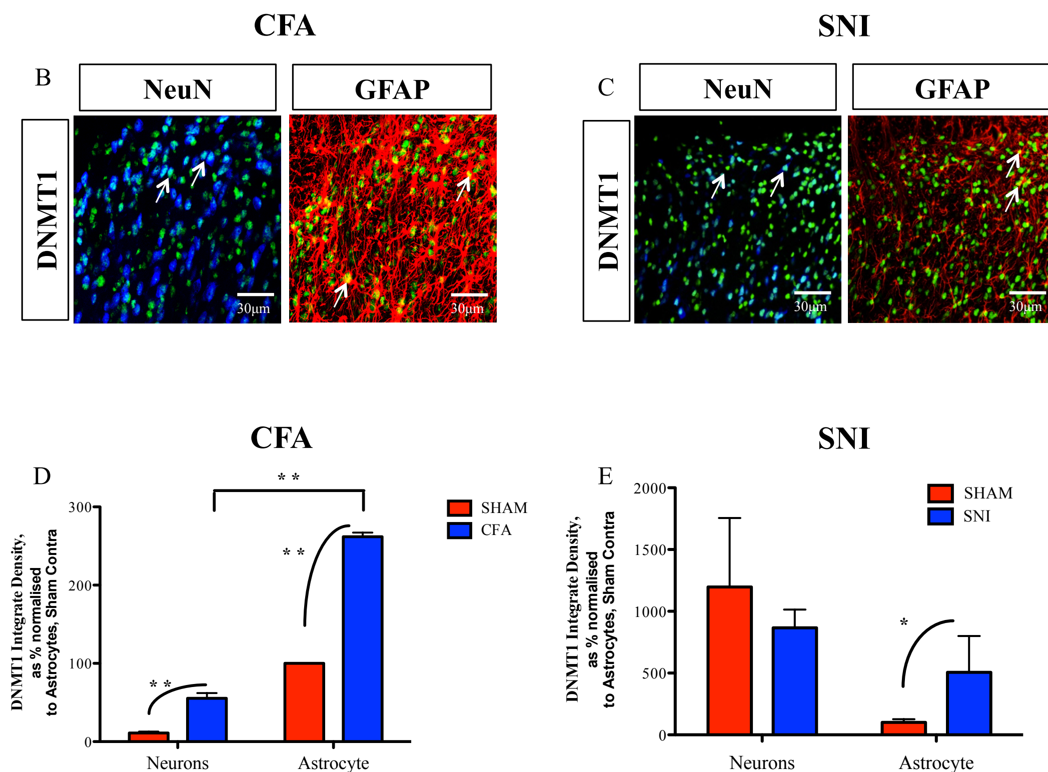

Fig. 7.13: DNMT1 expression increases in the superficial dorsal horn 7 days following SNI surgery and CFA injection. (A) Confocal images of rat spinal cord after SNI and CFA treatment; colocalization of DNMT1 (green), GFAP (red) and NeuN (blue); pictures show single focal plans; **(B) (C)** Higher magnification of A showing that DNMT1 can be seen within neurons and astrocytes **(D) (E)** analysis of DNMT1 integrate density with NeuN and GFAP following CFA and SNI. Signals are normalized against astrocyte contra sham. (n=4 for each group, *p<0,05, **p<0,01).

b. Setup of a protocol for TET1 detection and identification of its cellular localization in the spinal cord by immunohistochemistry.

The Ten-eleven translocation (Tet) family of enzymes has recently been implicated in DNA demethylation (Tahiliani et al, 2009). Also, Tochiki and colleagues (2012) have recently showed that a subset of protein involved in DNA methylation are downregulated 2h after CFA injection and suggested that this could be implicated in the upregulation of gene expression observed in the initial phase of this model of persistent pain. Another hypothesis could be that protein involved in DNA demethylation are upregulated 2h following CFA injection. To test this hypothesis, we set up a protocol for the detection of this protein. Our results showed that TET1 is uniformly distributed in the spinal cord (Fig 7.14, 10X) and mainly expressed in NeuN positive cells (Fig 7.14, Zoom) (arrows).

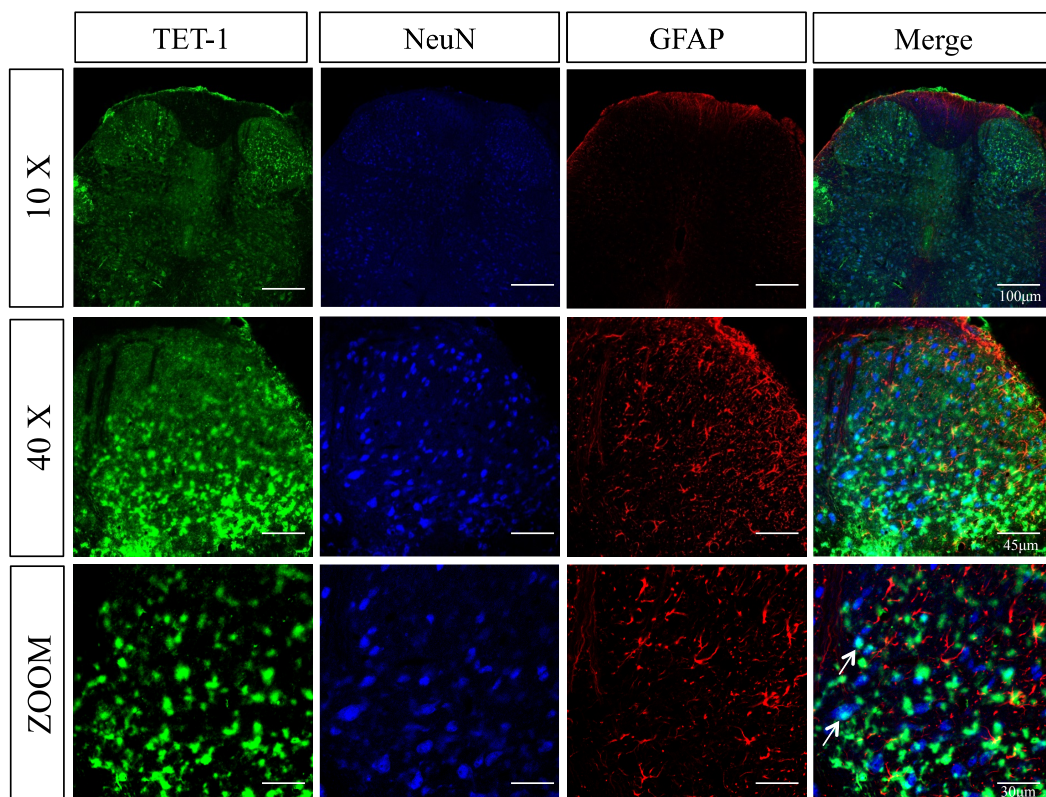

Fig 7.14: Cellular characterization of TET1 in the spinal cord. Confocal images of rat spinal cord sections. Colocalization of TET1 (*green*) and GFAP (*red*) and NeuN (*blu*). Picture show single focal plane.

8. DISCUSSION

Chronic pain is a very important problem because impairs everyday activities, social functions, quality of life and carrys great economic costs for society. Recently, IASP highlighted that the financial cost of chronic pain is roughly the same as cancer or cardiovascular disease. Moreover, the available treatments are only symptomatic and in the last 20 years no real advancement has been achieved in the pharmacological management of pain.

It is, therefore, mandatory a better understanding of the pathophysiological mechanisms underlying chronic pain for the development of new drug therapies.

Chronic pain states have been attributed to maladaptive molecular mechanisms at multiple levels through the pain system, for example at the level of the spinal cord and/or of the brain. We focus our attention on the spinal cord level and investigate the role of the autophagic process and epigenetic mechanisms in pain processing at this level.

Autophagy and pain

In pain condition, the occurrence of alteration in the autophagy process has been reported for the first time by our group (Berliocchi et al, 2011). In this thesis, we further extended our previous observation by characterizing the autophagic process in models of neuropathic and inflammatory pain.

To investigate whether autophagy modulation at spinal cord level may be relevant for pain processing, we analysed the autophagic markers LC3, Beclin 1 and p62 in three distinct models of partial peripheral nerve lesions: Spared Nerve injury (SNI; Decosterd and Woolf, 2000), Chronic Constriction Injury (CCI; Bennet and Xie, 1988) and Spinal Nerve Ligation (SNL; Kim & Chung, 1992).

Seven days after SNL and CCI and 14 days after SNI, a strong up-regulation of $\alpha_2\delta$-1 was observed in the ipsilateral spinal cord. The expression of this calcium channel subunit is dramatically increased in DRG and spinal dorsal horn following SNL surgery (Bauer, 2009). This overexpression occurs mainly in the side ipsilateral to the lesion and exclusively in the L4-L5 lumbar portion, thus providing a useful tool for identifying possible variability due to errors in sectioning of the very small sample. Although, for this work the dissection technique was highly improved in respect to

past studies (Berliocchi, 2011) and only the dorsal quadrant not the whole ipsilateral portion, of the lumbar L4-L5 cord segment was dissected and analysed, we still used the $\alpha_2\delta$-1 subunit as a biochemical marker for a state of neuropathic pain.

Microtubule-associated protein light chain 3 (LC3) is widely used to monitor autophagy (Klionsky et al, 2012). LC3 exist in two forms: LC3-I is the cytosolic unconjugated form that, after conjugation to phosphatidylethanolamine, is recruited to the autophagosomal membrane to form LC3-II (Galluzzi et al, 2009). LC3-II formation is correlated with the number of autophagosomes, and for this is considered one of the most reliable indicators of autophagosomes formation (Kabeya et al, 2000). In our study, the analysis of LC3 levels after SNL and SNI surgery revealed an important increase of this protein in the spinal dorsal horn ipsilateral to the injury. In particular, LC3-II increase was accompanied by a slight increase in LC3-I expression. No difference of expression between ipsi and contralateral side was observed after CCI surgery.

As already said, LC3-II is located to autophagosomal membrane and then degraded after fusion with lysosome, for this reason the analysis of LC3 levels has become a reliable method for monitoring autophagy (Klionsky et al, 2012). However, autophagosomes accumulation does not always mean induction of autophagy. Indeed, this accumulation may also be due to an increase in autophagic sequestration or to a blockade in autophagic flux (Mizushima et al, 2010). Also, deficits in the late steps of autophagy, especially at the level of autophagosome clearance following fusion with lysosome, may lead to autophagosome accumulation (Mizushima et al, 2010).

In order to have a wider view on autophagy modulation under our experimental conditions, we analysed the levels of two other proteins involved, respectively, upstream and downstream the autophagic flux: Beclin 1 and p62.

Evidence on the regulatory role of Beclin 1 on autophagy was obtained from study on yeast, where was shown that Beclin 1 complex is involved in autophagosome formation at an early stage (Pattingre et al, 2008). This complex is essential for the recruitment of other Atg proteins to the pre-autophagosomal structure (Suzuki et al, 2007). Our data show only a trend to increase of Beclin 1 expression in all three models of neuropathic pain used, suggesting that the observed upregulation of LC3-II may not be due to an enhanced induction of autophagy. The analysis of p62 expression became crucial. The ability of cells to degrade damaged organelles and

protein- aggregates may be protective against neurodegeneration (Mizushima, 2007). The protein p62 is a common marker for aggregates (Komatsu et al, 2006) and it accumulates in cells when autophagy is inhibited (Komatsu et al, 2006). Large aggregates, which include p62 and ubiquitin, have been identified in various neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Zatloukal et al, 2002). Several studies have showed that inhibition of autophagy is correlated with increased levels of p62 (Komatsu et al, 2007; Nezis et al, 2008). Similarly, decreased p62 levels are associated with autophagy activation (Wang et al, 2006). Our results showed p62 accumulation after SNI and SNL surgery, strengthening the hypothesis of autophagy impairment in these models in the clearance of autophagosomes. However, no difference of expression between ipsi and contralateral side was observed after CCI surgery. Because proteins can be degraded via autophagy and also via UPS-system, experiments were also done to assess the involvement of the proteosomal system in our experimental conditions, but results obtained did not provide conclusive information (data not shown).

While autophagic markers resulted modulated at the ipsilateral spinal dorsal horn following SNL and SNI, no relevant changes were observed in the sciatic CCI model. In general, all three methods of peripheral nerve injury produce behavioural signs of both ongoing and evoked pain with similar time course, as shown by a direct comparison (Kim et al, 1997). However, the models bear basic anatomical differences that can affect some of the physiological and biochemical changes underlying nociceptive and pain behaviour. Indeed, considerable difference in the magnitude of each pain component has been reported between models (Kim et al, 1997; Dowdall et al, 2005). For instance, signs of mechanical allodynia are largest in the SNL injury and smallest in the CCI model, whereas behavioural signs representing ongoing pain are much more prominent following CCI (Kim et al, 1997).

In the CCI model, the application of three sutures on the sciatic nerve determine the formation of intraneural oedema caused by the partial constriction of the vasculature of the epineurium with the appearance of demyelinated constrictions of 25-75% of the original diameter of the sciatic trunk (Bennet and Xie, 1988). This area merge over few days into a single area of uniform thinning, with the appearance of swelling proximal to the constricted area, referred as possible resistance to axoplasmic transport from the soma (Ossipov et al, 2013). An inflammatory reaction develops in

response to the sutures and consequently a loss of most A-fibres and some C-fibres, but few cell bodies (Tandrup et al, 2000). In the mouse, the SNL model consists of a specific injury (ligation) to the 5th lumbar segment (L5) spinal nerve distal to the DRG (Kim & Chung, 1992). This allows the manipulation of intact nerves or corresponding segments distinct from the injured ones and indeed this model allowed investigation on the importance of input from uninjured afferents in neuropathic pain (Li et al, 2000). In comparison to the CCI model, a more significant involvement of the sympathetic nervous system component in the sensory response has been described following SNL (Kim et al, 1997). Unlike the rapid sympathetic axonal sprouting observed after SNL (Ramer et al, 1998) and CCI (Ramer and Bisby, 1997), sprouting into the DRG after SNI is likely to contribute only to the maintenance, rather than the onset of neuropathic pain behaviours (Pertin et al, 2007). The SNI model involves a tight ligation and lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact. Therefore it differs from the SNL and CCI models in that the co-mingling of distal intact axons with degenerating axons is restricted (Decosterd 2000).

It is becoming more clear that SNL, SNI and CCI have distinct features not only in relation to manifestations of pain-like behaviour, but also to the underlying molecular and cellular mechanisms likely influenced by the different anatomy of the injury. Similarly, a widely used model of acute inflammatory pain like the formalin test is emerging as a model of long-lasting sensitization (Fu et al, 2001). Our data on the modulation of beclin1, LC3 and p62 over at different time points after formalin injection in the hind paw seems to be in agreement with this. Indeed, spinal autophagy seems to be inducted 30min and 2h after stimulation as shown by LC3 and p62 increase. However, p62 accumulation seems to be only transient as it disappeared from the ipsilateral side 4 days after injury. Moreover, at this time point the modulation of all autophagic markers is extended also to spinal cord side contralateral to injury. This seems to well fit with work from our group (data not shown) and others showing at these same time points a marked mechanical allodynia is present on both the injured and uninjured hind paw (Fu et al, 2001; Viercke et al, 2008; Ambriz-Tututi et al, 2009). Further work will be needed to understand the exact role of autophagy in these different experimental settings of neuropathic and inflammatory pain and will take advantage of the differences between models. However, to address the question whether autophagy modulation at the spinal cord is only an

epiphenomenon of the many changes occurring during central sensitization or whether autophagy can be a relevant mechanism for pain processing, we locally blocked autophagic flux at the lumbar spinal cord by intratechally injecting the autophagy blocker chloroquine in naïve mice. Chloroquine inhibits autophagy acting at lysosomal level (Klionsky et al, 2012) and can be used to both inhibit endogenous protein degradation and to increase the number of autophagic compartments (Klionsky et al, 2012). By elevating the lisosomal pH, chloroquine causes the inactivation of lisosomal enzymes and the accumulation of sequestered material that will be not degraded (Klionsky et al, 2012). In our experimental settings, we first confirmed by western blot the block of autophagic flux and showed the accumulation of p62 in chloroquine-treated mice, compared with vehicle-injected mice. Furthermore, we showed with behavioural tests that in the presence of spinal block of autophagy mice developed a drastic reduction in threshold of mechanical sensitivity, thus supporting our hypothesis of a relevant role of the autophagic process in spinal pain processing. Preliminary experiments have shown no signs of toxicity/cell death after chloroquine injection (data not shown) and further studies are on going to identify the position of autophagy within the chain of events participating to central sensitization.

Recently Pavone's group demonstrated that a reduction of autophagic activity in Schwann cells could be an early event in the chronification of neuropathic pain supporting the protective role of autophagy in the nervous system (Marinelli et al, 2013). Also in models of nerve injury, was demonstrated that the activation of autophagy can produce neuroprotective effect, reducing motor deficit observed in this condition (Tang et al, 2013; Hou et al, 2013). Conversely, Zhang and colleagues showed that inhibition of autophagy reduced the pain behaviour induced by SNL (Zhang et al, 2013). These data could seem at odds with our results. However, in this paper, the authors demonstrated an improvement of pain behaviour observed after SNL, through the inhibition of autophagy obtained by administering 3-MA, that inhibits autophagy upstream, blocking the step of sequestration (Klionsky et al, 2012). Instead, our data show that after nerve injury there is an activation of autophagic flux, as revealed by increased levels of LC3-II, but an impairment of the pathway downstream, in the process of degradation of autophagosomes, revealed by up regulation of p62 observed in SNL and SNI models and supported by reduction of

threshold of mechanical sensitivity observed after CQ injection in naïve mice. Moreover, Zhang and colleagues did not analysed p62 levels.

Since it is not clear at what step autophagy block occurs, if during induction, maturation or degradation of autophagosome, preliminary experiments were conducted to investigate lisosomal state by using the lysosomal marker LAMP (data not shown), that regulates the fusion of lysosomes with other organelles and is important for progression of autophagy (Saftig, 2005). Some evidence in the literature obtained from studies on cellular dysfunction in pancreatitis, indicate that defective lisosomal enzymes processing/activation leads to inefficient lisosomal degradation and retarded autophagic flux (Gukovsky et al, 2011; Mareninova et al, 2009; Gukovsky et al, 2010). Furthermore, growing attention is focussed to the lisosomal system especially for the emerging role of autophagy in a broad range of diseases (Futerman et al, 2004). Moreover, an increasing numbers of gene mutations has been identified that implicate lisosomal system dysfunction directly in adult neurological diseases (Nixon et al, 2008). However, only electron microscopy (EM) studies will provide valuable information on autophagosomal and lysosomal state and helping in identifying the nature of the block occurring at the spinal cord following peripheral nerve injury.

Furthermore, EM studies will help to understand where this block may occur, clarifying what late step of autophagy is involved. Evidence from literature suggest that autophagy dysfunction associated with autophagosome accumulation in neurons may be dependent by different process, as impaired vesicular transport (Stokin et al, 2005), defective fusion of autophagosomes with lysosomes (Ravikumar et al, 2005), reduced lisosomal activity (Bi et al, 1999). Using CQ we reduced lisosomal activity, inactivating lisosomal enzyme, then reducing autophagosome clearance.

Moreover, abnormal autophagic activity has been described in many human CNS-related disorders (Rubinsztein et al 2006; Winslow and Rubinsztein, 2008; Nixon et al, 2008), and recent studies provide evidence that autophagy may be protective in different neurodegenerative disease. Here the accumulation of autophagosome may represent on attempt of cell to activate autophagy as a beneficial physiologic response or the consequence of a defect in autophagosomal maturation, as observed for example in Alzheimer's disease (Martinez-Vicente et al, 2007, Rubinsztein et al, 2007; Williams et al 2008). The active role played by autophagy in neurodegeneration has been confirmed after the production of autophagy-deficient animal models. In

neuronal-specific knockout models of autophagic genes were observed signs of neurodegeneration, including progressive motor deficits, ataxia, growth retardation and also premature death (Nishiyama et al, 2007; Hara et al, 2006; Komatsu et al, 2006). The most observed feature in autophagy-defective neurons is the progressive accumulation of ubiquitin-positive inclusion bodies consistent of protein aggregates and damaged organelles that can interfere with cellular function and eventually lead to cell death.

Growing experimental evidence suggest that autophagy might play an important protective role in several neurodegenerative disorders such as Alzheimer's disease (Nixon et al, 2000; Pickfors et al, 2008), Parkinson' s disease (Michiorri et al 2010), Huntington's disease (Yamamoto et al, 2006) and epilepsy (Cao et al, 2009), where pharmacological stimulation of autophagic flux constitutes a promising clinical strategy for treatment.

Impaired autophagy may often lead to cell death. However, in the case of pain the contribution of spinal cell death to central sensitization is still very controversial. While apoptosis is reported to be responsible for the selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord, it is still questioned whether this cellular loss is necessary for the development of pain behaviour in several *in vivo* models of neuropathic pain (Scholz et al, 2005; Polgar et al, 2005; Polgar et al, 2003). Moreover, research so far has focussed on only one mode of cell death, namely apoptosis, without considering other neurodegenerative mechanisms, which are clearly playing an important role in other pathological conditions. Only recently the involvement of autophagy in neuropathic pain has received increased attention. Disregulation of autophagy may affect cell health, which is subject to a dynamic control by different players which the precise role to keep the cell functional (Bell and Hardingham, 2011; Isacson, 1993). Disruptions at the level of this control system may lead to a dysfunctional neuron and alter the neuronal circuitry. A block of autophagy can, therefore, impact neuronal function (Berliocchi et al, 2011).

The protein p62 is the key autophagic marker in our work and our results, obtained using immunohistochemistry methods, showed that p62 expression is mainly neuronal, and that only few GFAP-positive cells are also p62-positive and no colocalization of p62 was observed with the microglial marker Iba-1. However, other studies have showed just activation of autophagy also in microglia (Kanno et al, 2008; Shi et al, 2013). A main difference with our study is the autophagic markers used.

Kanno and colleagues showed an enhanced expression of Beclin 1 both in neurons and in glial cells, while Shi et al showed immunofluorescence images of LC3-II. However, for what we know, there are no antibodies that can discriminate between the two forms of LC3 (LC3-I and LC3-II) that can only be distinguished by their different in mobility in SDS-PAGE experiments. Moreover, our immunoistochemical results showed that p62 is strongly expresses in cell bodies, but it can be also detected in the neuropile opening the way to future investigations, suggesting that probably not only the neuronal soma is involved in autophagic impairment. Autophagy could be blocked along the axons but also at the synaptic terminals. A recent work by Tan and colleagues showed that the protein Rac-1 contribute to neuropathic pain by remodelling of dendritic spine (Tan et al, 2011). Additionally, Rac-1 activation delays autophagic flux and inhibition of this protein is essential for autophagic activation (Carroll et al, 2013), suggesting that autophagic flux could be blocked during neuropathic pain states, supporting our results. A further characterization of the spinal neuronal subtype needs to be done to understand wheter specific neuronal populations are more dependent on autophagy than other. Crucial experiment will be the analysis of p62 colocalization with markers for GABAergic neurons, because is know that the loss of GABA inhibitory tone is an important contributor for the development of pain (Hwang et al, 1997; Malan et al, 2002; Sivilotti and Woolf, 1994) and, recently, Chung's group have further demonstrated the involvement of this neuronal population in pain processing (Yowtak et al, 2013).

For the second part of this thesis, the research was carried out at the UCL in London, working with Doctor Sandrine Géranton. The work was focussed on the study of epigenetic mechanisms and the gene FKBP5 and their role in the modulation of pain behaviour. It is known that there may be interindividual differences to the response to pharmacological pain therapy and that these differences may be due to different genetic dispositions to develop pain or to respond to analgesic. The phenotype of a patient may be influenced by different factors; in particular, epigenetic mechanisms are very important because they can influence the translational state of a gene without altering its DNA sequence and determine, thus, that an identical combination of gene can produce a different phenotype (Doehring et al, 2011).

Growing evidence suggest an important role for epigenetic mechanisms in the control of pain states (Géranton 2012; Doehring, 2011). However, only few studies have

provided significant information regarding the contribution of epigenetic mechanisms on the modulation of gene expression in pain states. The information gained from previous molecular pain studies has provided a launch pad for further studies on the role of epigenetic mechanisms in the pain field. First, it has been shown that the process of injury and inflammation determine the release of substances which, after binding specific receptors, induce the activation of pathways that may ultimately affect gene expression (Donnerer et al, 1992; Sahbaie et al, 2009; Woolf et al, 1994). Second, microarray studies have analysed gene changes in different models of pain and provided lists of target genes that can be studied; for example substantial investigation has been done on opioid receptors (Hwang et al, 2010; Wei et al, 2011). It is also important to consider that epigenetic mechanisms regulate important events that may affect pain processing (Géranton, 2012): long-lasting neuronal plasticity is essential for the development of chronic pain and requires epigenetic modifications (Borelli et al, 2008; Sweatt, 2009); epigenetic modifications are essential for long-term memory formation that shares common mechanisms whit pain processing (Roth, 2009); finally, important signalling pathways and neurotrophins, involved in pain processing, induce epigenetic modification (Riccio, 2010).
Hunt and Géranton's work has provided important information about the regulation of gene expression in the spinal cord after noxious stimulation. In particular, they showed that a subset of genes is upregulated 2h after CFA injection in the ankle joint of rats, and demonstrated the involvement of the gene repressor MeCP2 in this process (Géranton, 2007). My attention was focussed on one of these genes, the FKBP5 gene. Moreover, recent evidence demonstrated that this gene is under strong epigenetic regulation (Klengel et al, 2013).

The gene FKBP5 is an important regulator of the response to stress and, growing evidence suggests that the state of mind, such as chronic stress, can modulate pain sensation (Wagner, 2013). Recent research has indeed shown that stress can have a number of effects on pain perception (Rhudy and Meagher, 2000; Meagher et al, 2001): while extreme stress can produce "stress-induced analgesia" therefore inhibiting pain, mild or prolonged stress usually enhances pain sensation, producing "stress-induced hyperalgesia". While FKBP5 expression has been studied in different conditions of state of mind, such as depression and post-traumatic stress disorders

(O'Leary et al, 2011; Klengel et al, 2013), the involvement of FKBP5 in pain state has never been investigated.

FKBP5 effect on pain behaviour

In this study we demonstrated that the gene FKBP5 is involved in the maintenance, but also in the induction phase of chronic pain, opening a new road to a better understanding of pain mechanisms.

For our study, we have had the opportunity to use non-commercially available FKBP5 -/- mice. These mice are used only in three labs, and we are the only pain specialists studying pain behaviour in these animals.

Others have shown than these mice show more active stress-coping behaviour than naïve mice, anti-depressant behaviour, but no impairment of motor and cognitive functions (O'Leary et al, 2011). Our hypothesis was that they would display less pain sensitivity.

In different models of acute pain, where the painful sensation lasts a short time, we didn't observe any difference in pain behaviour between FKBP5 -/- and FKBP5 +/+ mice. Immunohistochemical analysis of c-Fos expression in the spinal cord of both genotypes confirmed that there was no difference in the total neuronal activation in the dorsal horn in these models.

Important differences were, however, observed in different models of chronic pain.

The injection of CFA in the ankle joint induces a drastic reduction of threshold to mechanical stimulation. This phenomenon, called mechanical allodynia, starts few hours after the injection and remains constant for at least one week. When injected with CFA in the ankle joint, FKBP5 -/- mice showed less sensitivity to mechanical stimulation than FKBP5 +/+ mice. This was also observed using a model of neuropathic pain: the spared nerve injury model. This model was performed according to Decosterd and Woolf (2010), and induces a robust mechanical allodynia that develops few days after surgery and remains constant for several weeks. Only an initial reduction of threshold of mechanical sensitivity was observed in FKBP5 -/- that never reached the max sensitivity seen in FKBP5 +/+ mice. Another important result was also the difference observed in locomotion following SNI surgery. It is known, and also observed in clinical conditions, that nerve injury induces a reduction of motor functions (Urban et al, 2011). Our results showed that, in mice that had

undergone SNI surgery, deletion of the FKBP5 gene improves this deficit. Indeed, the FKBP5 -/ mice could stay on the Rotarod longer than wild type mice and for times comparable to naïve mice. This difference was not observed 3 days after surgery, when there was no difference in the mechanical sensitivity between FKBP5 -/- and FKBP5 +/+ animals, but only once the difference in the threshold of mechanical sensitivity had become significant. Finally, enhanced sensitivity to cold stimulation is often observed in clinical condition, but we didn't observed any difference between FKBP5 -/- and FKBP5+/+ mice after stimulation with aceton. While we cannot explain these results yet, they could well be due to the low number of animals used in this study.

To investigate the reason behind the differences observed in pain behaviour, we looked at the glial activation in the spinal cord and at the activation of the ERK pathway in the rostral ventromedial medulla (RVM).

Neuronal cells are not the only cell type involved in chronic pain states. It has been reported that glial cells, both astrocyte and microglia, are also possible players in the initiation and maintenance phase of neuropathic and inflammatory pain (Milligan and Watkind, 2009). However, our results showed that 7 days after SNI surgery there were no differences in glial activation in the spinal cord between the two genotypes.

The RVM is a major source of descending pathways to the spinal cord and the inputs from this area exert both facilitatory and inhibitory influence on nociceptive transmission in the spinal cord (Basbaum and Fields, 1984). Moreover, the Mitogen Activated Protein (MAP) kinases are involved in pain processing: the extracellular signal-related kinase (ERK) plays important roles in synaptic plasticity and memory formation (Sweatt, 2001) and phosphorylated ERK (p-ERK), the active form of ERK, is a marker of neuronal activation. Experimental evidence demonstrated that this phosphorylated form of ERK was up-regulated in the RVM following CFA injection in the hind paw (Imbe et al, 2005) and inhibition of this signal within the RVM reduced thermal hyperalgesia after inflammation (Imbe et al, 2005; Imbe et al, 2008). However, following analysis of the expression of p-ERK in RVM (data not shown in this thesis) no differences were observed between the two genotypes. So far we have only looked at the most obvious pathway involved in pain modulation, and therefore further investigation will be needed to understand the underlying molecular mechanisms behind the differences in behaviour seen between the two genotypes.

Up to this point, our results suggest an important role for the gene FKBP5 in the maintenance phase of chronic pain with a strong influence on secondary hyperalgesia. Secondary hyperalgesia occurs in the injured tissue surrounding the site of injury (e.g. the hindpaw for the inflamed ankle joint and the side of the paw after SNI) and is thought to be due to sensitization in central nervous system. This type of hyperalgesia, characterized by enhanced sensitivity to mechanical, but not heat, stimuli, is comparable to the hyperalgesia seen in patients with neuropathic pain (Meyer et al, 2006). Primary hyperalgesia, however, occurs at the site of tissue injury and is mediated in part by sensitization of primary afferent nociceptors. Primary hyperalgesia is characterized by increased response to heat stimuli, for example (Meyer et al, 2006). To investigate whether the deletion of the FKBP5 gene influences also primary hyperalgesia, mice were subjected to intraplantar administration of CFA and mechanical and thermal threshold were measured in the area of injury, i.e. where the paw is swollen. No difference was observed in the thermal hyperalgesia between the two genotypes, suggesting that FKBP5 deletion might not influence primary hyperalgesia, or at least thermal hyperalgesia. Future experiments will be performed to look at c-Fos expression in the spinal cord following CFA injection in the hind paw, but we expect no difference between the two genotypes. On the contrary, we observed again an important difference in the development of mechanical allodynia, with FKBP5 -/- animals being less sensitive that wild type animals. However, this decrease was only 77% of that of wild type mice (when compared to baseline), much less that that seen with other models of persistent pain.

All together these data, confirmed our initial hypothesis since FKBP5 -/- displayed less pain behaviour after painful stimulation in model of long-term pain states. However, these mice are global knockouts, so it was important to examine the specific role of FKBP5 on pain processing at the level of the spinal dorsal horn. Géranton and colleagues showed that in the spinal cord, 2h after CFA injection, the transcription of this gene is enhanced, suggesting a possible role of spinal FKBP5 in the early phase of pain processing.

Others have successfully silenced genes in the spinal cord and shown their role in pain processing (Tan et al, 2005; Garraway et al, 2007; Dore-Savard et al, 2008). Using antisense technology, we silenced the gene FKBP5 only in the spinal cord while allowing its expression in the rest of the body. siRNA was injected intrathecally

in wild type mice for three consecutive days, three days after CFA injection in the ankle joint, once the mechanical allodynia was completely developed. Starting from the second injection, the siRNA-mice displayed less sensitivity to mechanical stimulation than vehicle injected mice. This result supports the idea of the involvement of FKBP5 on pain processing in the spinal cord and, also, confirmed the role of this gene in the maintenance phase of chronic pain.

Another important result was obtained with the silencing of the gene before the painful stimulation. Wild type mice received siRNA for three consecutive days, with CFA injected on the day of the third injection. siRNA mice developed a lower degree of mechanical hypersensitivity after CFA when compared to vehicle-treated mice that received only vehicle. This experiment has added another element to the understanding of the role of FKBP5 gene in pain processing. It confirmed the importance of the expression of this gene in the spinal cord for pain processing and suggested an important role also in the induction of the pain state. This was supported by the original data from Géranton et al. (2007) reporting an increase in FKBP5 expression in the dorsal horn 2h post CFA injection.

Silencing of FKBP5 before noxious stimulation could therefore reduce the insult produced by CFA injection.

It is important to note that the injection of antisense did not influence the baseline of threshold of mechanical sensitivity and that no change was observed in the contralateral side of siRNA-injected mice.

Experiments are ongoing to check the effective expression levels of the protein.

FK506 binding protein 51, is a 51-kDa protein encoded by the FKBP5 gene located on the short harm of the human chromosome 6 (Nair et al, 1997). This protein is an important regulator of the GR-complex (Grad et al, 2007). Several experimental evidences have shown an important role of GR in the mechanisms of inflammation and chronic pain (Neeck et al, 2002; Wang et al, 2004). In particular Wang and colleagues observed that after peripheral nerve injury a time-dependent expression of neuronal GR is seen in the spinal cord ipsilateral to the injury; moreover, the administration of GR antagonists attenuated the development of thermal hyperalgesia and mechanical allodynia typically observed in neuropathic pain condition (Wang et al, 2004). FKBP5 directly acts on GR activity by altering glucocorticoid receptor function by decreasing ligand binding and impeding translocation of the receptor

complex to the nucleus (Wochnik et al, 2005). It is probable that the inhibition of GR, through the use of antagonists, might induce a downstream reduction in transcription of the gene FKBP5. At this point of our research it remains very complicated to explain the mechanisms behind the difference observed and further investigations will be carried out also to better characterise the state of the GR system in the FKBP5 -/- mice.

Here we have shown for the first time that the ablation of the FKBP5 gene in mice leads to reduced pain sensitivity in different models of chronic pain. Also, silencing of FKBP5 only in the spinal cord provided important information about the role of this gene in pain processing. Moreover, because others have previously shown that FKBP5 ablation results in reduced anxiety-like behaviour in mice (O'Leary et al, 2011), our study supports the notion that different state of mind, in particular stress, may influence pain behaviour.

Although our data cannot explain the mechanisms behind our observations yet, they open a new line of investigation and further molecular analysis will be performed to better understand the underlying mechanisms.

DNMT1 expression during the maintenance phase of persistent pain

DNA methylation is a major epigenetic mechanism regulating gene expression and DNA methyltransferases (DNMTs) are the main enzymes involved in this process. In this part of the study, work was focussed on the analysis of DNMT1 expression, enzyme responsible of *de novo* DNA methylation.

A previous study performed by Tochiki and colleagues showed that 7 days following CFA injection in the ankle joint there was an increase in mRNA levels for DNMT1 within the dorsal horn, but a decrease following SNI (Tochiki et al, 2012). However, this evidence was based on qPCR results, without any information about the cell type involved in this process. Here, analysis of DNMT1 expression in neurons and in astrocytes 7 days after SNI showed an important upregulation of DNMT1 expression in astrocytes suggesting that this enzyme could be more important in this cell type than in neurons in this model of chronic pain. These results differ from previous result published by Tochiki et al., who showed a small ipsilateral downregulation of DNMT1 mRNAs 7 days after SNI surgery compared to the upregulation of protein that we observed. This difference could be explained in different ways. Firstly,

Tochiki and colleagues used RT-qPCR to quantify mRNA levels of DNMT1 as opposed to protein levels as measured by immunohistochemistry. Very often, conclusion about protein expression is based only on mRNA analysis. However, it is accepted that change in mRNA levels are not always reflective of changes in protein expression. For instance, this has been recently reported by Shebl and colleagues (2010) in the case of cytokine and chemokine mRNA levels to protein expression. Géranton group showed the analogous differences in HDAC-2 expression where analysed by qPCR or immunohistochemistry (unpublished data). Therefore, our data confirm that change in mRNA levels do not always reflect similar changes in protein expression.

In CFA-injected mice, however, a significant upregulation of this enzyme was observed in both neurones and astrocytes. These results supported the previous results from Tochiki et al. (2012).

Our results therefore suggested a different role of DMNT1 in these two models of chronic pain, laying the groundwork for future investigation about the role of this enzyme in changes in methylation of DNA after noxious stimulation.

Previous work has indicated that gene transcription is enhanced 2h following CFA injection (Géranton et al, 2007). These results could also suggest the involvement of other proteins in active demethylation of DNA. In particular we hypothesised that the protein TET1, recently identified as the main player in DNA demethylation (Tahiliani et al, 2009), could have an active role in the process. TET1, converting 5mC to 5-hydroxymethylcytosined (5hmC) promotes DNA demethylation thus influencing gene transcription.

Future experiments will be performed to analyse the expression levels of this protein in the spinal cord after noxious stimulation and to explore its role in the upregulation of gene expression observed in the initial phase of persistent pain states (Tochiki et al, 2012).

FKBP5 and its epigenetic regulation

Recent experimental evidence showed that FKBP5 is under strong epigenetic regulation. In particular, Klengel and colleagues demonstrated that demethylation of this gene is linked to increased stress-dependent gene transcription. In this work, the author demonstrated that FKBP5 can prime the stress response. Considering the evidence obtained in our experiments showing an active role of FKBP5 in spinal cord

pain processing, future investigation will be focused on the study of the role of this gene as primer for pain response.

FKBP5 -/- mice will be used in experimental procedure based on the *Hyperalgesic priming* protocol, to understand if the deletion of this gene may influence the development of prolonged hypersensitivity observed in this model.

9. CONCLUSION AND FUTURE PERSPECTIVES

Both research projects approached two emerging cellular processes that had not been extensively studied in the field of pain yet. Part of the work was therefore dedicated to set up appropriate models together with appropriate experimental tools, and to characterize both autophagic and epigenetic mechanisms in different models of pain. the data collected, although not always conclusive, opened new opportunities in the identification of new mechanisms and therapeutic targets and provide a valuable basis for future investigations.

Autophagy and pain

All together our data extended our previous work and opened new lines for further investigation on the role of autophagy in pain processing. Understanding the exact role of autophagy in this process may lead to the identification of new pharmacological target and the development of new drugs or, alternatively, lead to new indications for existing drugs know to modulate some of the regulatory pathways of the autophagic process and currently used for different therapeutic applications.

FKBP5 gene and epigenetic mechanisms

Our results provide, for the first time, important evidence about the involvement of the gene FKBP5 in pain processing, provide important information about the enzymes involved in the changes in DNA methylation seen after noxious stimulation and the cell types involved in this process, and lay the basis for a better understanding of the pathophysiology of chronic pain states by including the gene FKBP5 and epigenetic modifications as target for the development of new pain treatments.

All together, these data have allowed us to improve out knowledge in the field of pain. Such knowledge and future investigations will open up opportunities for developing novel analgesics.

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11. PUBLICATIONS and CONTRIBUTIONS

✓ Publication

Berliocchi L., Russo R., **Maiarù M**., Levato A., Bagetta G., Corasaniti MT. Autophagy impairment in a mouse model of neuropathic pain. Mol Pain 2011, 7:83.

✓ Communications to International Meetings

1. **Maiarù M**. Antinociceptive properties of the essential oil of bergamot and linalool. NEW TREND IN PAIN RESEARCH. From Basic Research to clinical Translation. Parghelia (VV) (Italy), 13-15 September 2012. **(Oral Communication)**

2. **Maiarù M**, Berliocchi L, Levato A, Rombolà L, Morrone LA, Bagetta G and Corasaniti MT. Antinociceptive properties of the essential oil of bergamot and linalool. 14th World Congress on Pain. Milan (Italy), 27-31 August 2012.

3. Berliocchi L, Russo R, **Maiarù M**, Tassorelli C, Bagetta G. and Corasaniti MT. Spinal autophagy modulation following peripheral injury. 14th World Congress on Pain. Milan (Italy), 27-31 August 2012.

4. Berliocchi L, Levato A, Rombolà L, **Maiarù M**, Morrone LA, Bagetta G and Corasaniti MT. Proprietà antinocicettive dell’olio essenziale di bergamotto e del linalolo. La chimica della vita: da alimenti funzionali a principi attivi, Catanzaro (Italy), 29/30 September 2011.

5. Berliocchi L, Russo R, **Maiarù M** and Corasaniti MT. Autophagy in experimental models of pain. 35th National Congress SIF, Bologna (Italy), 14-17 September 2011.

6. Berliocchi L, Russo R, **Maiarù M**, Levato A, Bagetta G and Corasaniti MT. Autophagy disruption in the mouse spinal cord following peripheral nerve injury. 8th World Congress of neuroscience. Firenze (Italy), 14-18 July 2011

7. **Maiarù M**, Berliocchi L, Bagetta G, Corasaniti MT. Gender differences in pain response to peripheral injury in mice. 8th World Congress of neuroscience. Firenze (Italy), 14-18 July 2011.

8. Berliocchi L, Russo R, Levato A, **Maiarù M,** Bagetta G, Corasaniti MT. Expression of the autophagic marker LC3 and beclin in the spinal cord of mice subjected to ligation of the L5 spinal nerve. 34th National Congress AISD, Riccione (Italy) 29-31 May 2011.

9. **Maiarù M.**, Russo R., Levato A. , Rombolà L. , Mizoguchi H., Bagetta G., Corasaniti M.T., Berliocchi L. Spinal nerve ligation promotes spinal expression of the autophagic markers LC3 and beclin-1. Monothematic meeting Cellular and molecular aspect of Pharmacologic control of pain, Parghelia (Italy) 22-24 September 2010. (**Oral Comunication**).

✓ Awards

1. Nine months travel grant from **SIF** (Società Italiana di Farmacologia) for stage in London

2. Travel grant from **SINS** (Società Italiana di Neuroscienze) for participation to International meeting

SOMMARIO

Il dolore è una "spiacevole esperienza sensoriale associata a danno tissutale, descritta in termini di tale danno" (IASP, 2011). Il dolore fisiologico è importante per evitare potenziali danni, mente il dolore patologico è sgradevole, permanente per un periodo di tempo anche dopo il danno ed è caratterizzato da una maggiore reattività a stimoli sia nocivi che innocui (iperalgesia ed allodinia). Il dolore acuto ha una funzione difensiva: evita nuovi stimoli su una parte già lesa e si risolve entro pochi giorni, mentre il dolore cronico può perpetuarsi nel tempo. Il dolore neuropatico viene definito come uno stato di dolore cronico risultante da un danno a livello del sistema nervoso centrale o periferico. Al momento i trattamenti farmacologici disponibili per il dolore neuropatico, inclusi gli antidepressivi triciclici e l'attuale "gold standard" Gabapentin, solitamente hanno una efficiacia limitata nella maggior parte dei pazienti (Childers et al, 2007). Sviluppare un miglior trattamento per il dolore neuropatico e soprattutto una migliore conoscenza della sua patofisiologia è quindi, molto importante. Al fine di migliorare le conoscenze dei meccanismi alla base degli stati di dolore è stato studiato il ruolo di due nuovi processi cellulari: l'autofagia ed i meccanismi epigenetici. L'autofagia è la principale via coinvolta nei meccanismi di degradazione delle proteine e degli organelli cellulari, nel rimodellamento cellulare e nella sopravvivenza cellulare durante i periodi di carenza di nutrienti. La diminuzione dell'attività autofagica sembra interferire con la degradazione delle proteine e con il turnover dei nutrienti, mentre una maggiore attivazione di questa pathway sembra facilitare la clearance di aggregati proteici e promuovere la sopravvivenza neuronale in varie malattie neurodegenerative. D'altra parte però, un'attività autofagica troppo elevata può essere dannosa e portare alla morte della cellula, suggerendo come un regolamento dell'autofagia abbia un ruolo importante nel determinare il destino cellulare. Tuttavia, nonostante i numerosi studi sul ruolo dell'autofagia nelle malattie neurodegenerative, il ruolo di tale processo nella fisiopatologia del dolore neuropatico rimane ancora poco studiato.

I meccanismi epigenetici sono quelle modificazioni chimiche della cromatina che influenzano l'espressione genica senza però alterare la sequenza del DNA. Nonostante negli ultimi anni la ricerca scientifica abbia prodotto importanti risultati

nel campo dell'epigenetica, solo pochi studi si sono concentrati sul coinvolgimento dei meccanismi epigenetici in relazione agli stati di dolore. Evidenze sperimentali suggeriscono che i cambiamenti dell'espressione di alcuni geni siano coinvolti nelle fasi di induzione e di mantenimento degli stati di dolore cronico. Tra questi geni, recenti evidenze suggeriscono un ruolo per il gene FKBP5, importante regolatore del recettore per i glucocorticoidi e coinvolto nel sistema di regolazione della risposta allo stress. Inoltre, recenti studi dimostrano come questo gene sia sotto forte controllo epigenetico. Alla luce di tutto ciò, gli obiettivi del presente lavoro di ricerca sono stati:

- caratterizzare il processo autofagico a livello del midollo spinale, in diversi modelli sperimentali di dolore neuropatico e infiammatorio;
- valutare l'importanza del processo autofagico nei meccanismi di elaborazione del dolore;
- caratterizzare gli enzimi coinvolti nei processi di metilazione del DNA
- identificare le condizioni di dolore che possono essere alleviate in seguito al silenziamento del gene FKBP5

I risultati ottenuti nella prima parte sperimentale di questo lavoro di tesi, dimostrano che in diversi modelli sperimentali di dolore neuropatico l'espressione dei markers autofagici viene ad essere modulata. In particolare nel modello che prevede la legatura del nervo spinale L5 (SNL) e nel modello che prevede la recisione del nervo tibiale e peroneale (SNI), si è osservato un aumento della forma associata all'autofagosoma della proteina LC3 (LC3II) e della proteina p62, coinvolta nelle fasi di degradazione del processo autofagico. L'aumento osservato dei livelli della proteina p62 ha suggerito un possibile blocco del flusso autofagico. Nel presente lavoro è stato studiato l'effetto del blocco dell'autofagia sul comportamento dolorifico. In particolare, il trattamento di animali naïve con Clorochina, un inibitore lisosomiale, ha determinato l'instaurarsi di uno stato di iperalgesia tipicamente osservato in seguito a danno periferico dei nervi spinali. I risultati ottenuti nella seconda parte sperimentale dimostrano un coinvolgimento del gene FKBP5 nelle fasi di induzione e di mantenimento del dolore cronico. In particolare, animali knockout per questo gene hanno dimostrato una minore sensibilità agli stimoli meccanici in seguito all'instaurarsi di diversi stati di dolore cronico. Il silenziamento a livello spinale del gene ha permesso di comprendere il ruolo del gene FKBP5 nel processamento dell'informazione dolorosa in seguito a danno. Infine, lo studio e la

caratterizzazione di DNMT1, enzima coinvolto nella metilazione del DNA, ha permesso di suggerire il coinvolgimento attivo di altre proteine nel processo di demetilazione di DNA e quindi nell'espressione dei geni coinvolti. In conclusione i dati riportati in questo studio indicano un blocco del processo autofagico in modelli sperimentali di dolore neuropatico, supportando il ruolo neuroprotettivo di questo processo nel midollo spinale. Inoltre è stato dimostrato il coinvolgimento del gene FKBP5 nell'induzione e nel mantenimento di stati di dolore cronico. Pertanto, tutti insieme questi dati aprono la strada a ulteriori investigazioni per una migliore comprensione dei meccanismi alla basa del dolore cronico e quindi l'identificazione di potenziali bersagli molecolari per lo sviluppo di nuove strategie terapeutiche.