UNIVERSIDAD DE CANTABRIA
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Neuropathic pain induced by nerve injury involves epigenetic changes and chromatolytic damage in the somatosensory nervous system. Effects of miR-30c-5p gain and loss of function

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EXPONEN:

Que han llevado a cabo las funciones de dirección de la Tesis Doctoral de Dña **Raquel Francés Romero,** Licenciada en Biotecnología con el título: "Neuropathic pain induced by nerve injury involves epigenetic changes and chromatolytic damage in the somatosensory nervous system. Effects of miR-30c-5p gain and loss of function".

Y que dicho trabajo se encuentra terminado y reúne los requisitos necesarios para su presentación como Memoria de Doctorado, con el objeto de poder optar al grado de Doctor por la Universidad de Cantabria.

Y para que conste y surta los efectos oportunos, expedimos la presente certificación en Santander, a 8 de Enero de 2019.

Fdo. Mª Amor Hurlé González Fdo. Mónica Tramullas Fernández

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Index

Abbreviations

AGO: Argonaute

APS: Amonium persulafte

ATP: Adenosine triphosphate

BDNF: Brain-derived neurotrophic factor

bp: Base pairs

BSA: Bovine serum albumin

ºC: Celsius degrees

C.elegans: Caernohabditis elegans

$CaCl_2$: Calcium dichloride

CB: Cajal body

CCI: Chronic constriction injury

cDNA: Complementary DNA

CNS: Central nervous system

cm: Centimeters

CO_2: Carbon dioxide

COX: Cyclooxigenase

CSF: Cerebrospinal Fluid

Ct: Cycle threshold

DFC: Dense fibrillar component

DGCR8: Digeorge syndrome chromosomal or critical region 8 (microprocessor complex)

DICER: Double strand RNA binding domain

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethylsulphoxide

DNA: Deoxyribonucleic acid

DNMTs: DNA methyltransferases

DNMT1: DNA methyltransferase 1

DNMT3a: DNA methyltransferase 3a

DNMT3b: DNA methyltransferase 3b

DNMT3L: DNMT3-Like protein

dNTPs: Deoxynucleotide triphosphates

DRG: Dorsal root ganglia

DROSHA: Ribonuclease type III

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic adic

FBS: Fetal bovine serum

FC: Fibrillar center

FITC: Fluorescein isothiocyanate

g: Grams

GC: Granular component

h: Hour

H^+: Hydrogen molecule

HAT: Histone acetyltransferase

HCl: Hydrochloric acid

HDAC: Histone deacetylase

HDMs: Histone demethylases

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMTs: Histone methyltransferases

H_2O: Water

H_2O DEPC: Diethylpyrocarbonate water

H3K9me3: Histone 3 trimethylated in lysine 3

IASP: International Association for the Study of Pain

IF: Immunofluorescence

K: Lysine

K^+: Potassium molecule

KCl: Potassium Chloride

kDa: Kilodalton

KDM: Lysine demethylase

kg: kilograms

KH_2PO_4: Potassium phosphate monobasic

KMT: Lysine methyl-transferase

Lamb1: Lamina b1

M: Molar

m: Meter

MBPs: Methyl-binding proteins

mg: milligrams

Mg^{2+}: Magnesium molecule

$MgCl_2$: Magnesium chloride

microRT-PCR: Micro-retrotranscriptase PCR

miRISC: MiRNA-induced silencing complex

miRNA: MicroRNA

ml: Mililiters

mM: Millimolar

mm: Millimeters

mRNA: Messenger RNA

ms: Miliseconds

ms-qPCR: Methylation sensitive qPCR

mT: Melting temperature

MOR: Mu opioid receptor

Na^+: Sodium molecule

Na_2HPO_4: Sodium phosphate dibasic

NaCl: Sodium chloride

NaH_2PO_4: Monosodium phosphate

NB: Nissl bodies

nBLAST: nucleotide basic local alignment search tool

ncRNA: Non-coding RNA

Nfyc: Nuclear transcription factor Y subunit gamma

ng: Nanograms

nm: Nanometer

nM: Nanomolar

NMDA: N-methyl-D-aspartate recepot

No: Nucleolus

NO: Nitric oxide

NS: Nervous system

NSAIDs: non-steroidal anti-inflammatory drugs

O_2: Oxygen molecule

PAG: Periaqueductal gray

PBS: Phosphate buffered saline

PCR: Polimerase chain reaction

PFA: Paraformaldehyde

PI: Propidium Iodide

PNS: Peripheral nervous system

Pre-miRNA: MiRNA precursor

Pre-rRNA: Pre-ribosomal RNA

Pri-miRNA: Primary miRNA

PSL: Partial sciatic ligation

PVDF: Polyvinylidene difluoride

qPCR: Quantitative Polimerase chain reaction

R: Arginine

RAN-GTP: Ras-related nuclear protein Guanosine-5-triphosphate

rpm: Revolutions per minute

RER: Rough endoplasmic reticulum

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

RT: Room temperature

RT-PCR: Retrotranscriptase PCR

RVM: Rostral ventromedial medulla

s: Seconds

SAM: S-adenosyl-L-methionine

SDH: Spinal dorsal horn

SDS: Sodium dodecyl sulfate

SEM: Standard error mean

SI: Signal intensity

SNI: Spared nerve injury

SNL: Spared nerve ligation

snoRNPs: Small nucleolar ribonucleoproteins

snRNPs: Small nuclear ribonucleoproteins

Suv39h1: Suppressor of variegation 3-9 homolog 1

T: Temperature

TBS-T: Tris Buffer Saline- Tween 20

TGB-β: Transforming growth factor β

TNF: Tumor necrosis factor

TRBP: TAR RNA binding protein

TRPA1: Transient receptor potential cation channel subfamily A member 1

TRPM8: Transient receptor potential cation channel subfamily M member 8

U: Units

UBF: Upstream binding factor

UTR: Untranslated region

V: Volts

WB: Western blot

5'-hmC: 5'-hydroxymethyl cytosine

5-HT: Serotonin

5'-MeC: 5'-methylcytosine

µl: Microliters

µm: Micrometers

Justification and objetives

Pain is an unpleasant experience that alerts the body of actual or potential tissue damages or diseases. Physiological pain is proportional to the intensity of the stimulus that caused it and triggers protective responses directed to the defense of the organism. Pain usually disappears after the healing of the triggering lesion. However, in certain situations of neural damage or inflammation, pain may persist long after the healing of the lesion.

Chronic pain is a highly prevalent condition among the general population (~20 %), which constitutes a serious burden on life quality of patients and an important public health problem, with socio-economic and health related consequences. Chronic pain is considered a pathological process in itself, which requires specific treatment.

A particularly devastating form of pathological pain is neuropathic pain which arises as a consequence of a lesion or disease affecting the somatosensory system, peripheral or central. According to data from The Spanish Pain Society, the prevalence of neuropathic pain is estimated in 8–10 % of the general population in Spain. Common causes of neuropathic pain include, among others, metabolic (diabetes) and infectious (herpes zoster, VIH) diseases, traumatisms (surgery, spinal cord injury), tumors, neuralgias, stroke, peripheral ischemia, etc.

The therapeutic approach to neuropathic pain is basically focused on four pharmacological groups: antidepressants, antiepileptics, local anesthetics, and several opioids. However, management neuropathic pain is still a challenge because the response to pharmacological treatments is generally limited. Thus, the number-needed-to-treat, for 50% pain relief in one patient, ranges from 3 to 5 of patients. Moreover, the drugs currently available do not have the capacity to modify the evolutionary course of neuropathic pain, and lack preventive/curative properties.

Our incomplete understanding of the cellular and molecular mechanisms responsible for neuropathic pain establishment and chronification has limited the identification of novel therapeutic targets for its prevention and/or release. Therefore, their study is a priority field

In the recent years, a rapidly increasing body of evidence implicates epigenetic modifications, such as DNA methylation, histone modifications and

microRNAs (RNAs), in the long-lasting aberrant expression of crucial pain-related genes in the somatosensory nervous system, underlying pain chronification after neural injuries.

In this regard, our group has been studying for several years the role of miRNA-related epigenetic mechanisms involved in the persisting neural adaptations triggered by peripheral nerve damage, which contribute to the development of neuropathic pain. We have previously demonstrated a key role for miR-30c-5p in the development and persistence of neuropathic pain, as well as its potential value as biomarker and therapeutic target, both in an experimental animal model and in patients. Our preclinical study shows that the severity of neuropathic pain following spared nerve injury (SNI) in rats is directly related with miR-30c-5p overexpression in relevant regions of the nociceptive pathway. Consistently, the administration in the cisterna magna of miR-30c-mimic accelerates the development of allodynia. In contrast, the administration of a miR-30c-5p specific inhibitor, at the time of nerve injury, prevents the development of allodynia. But most importantly, the rats treated with miR-30c-5p inhibitor several weeks after allodynia is fully established recover the normal nociceptive sensitivity. In both cases, the animals remained free of neuropathic pain until their sacrifice, two months after nerve injury. Overall, our results in experimental animals point to a relevant contribution of miR-30c-5p to mechanical allodynia development, and make it a promising target for treating neuropathic pain states.

The relevance of these findings was transferred to the clinic, in a group of patients with ischemia of the lower extremities. The logistic regression analysis allowed us to develop a model in which the circulating levels of miR-30c-5p in CSF or in plasma discriminate with great sensitivity and specificity those patients who suffer from neuropathic pain of those who are free of pain.

Online software has revealed that the *de novo* DNA methyltransferase DNMT3b enzyme is found among the predicted transcript targets for miR-30c-5p.

Increasing evidence points to the involvement of epigenetic processes in the aberrant expression of genes encoding ion channels, receptors, neurotransmitters, modulators, cytokines, etc., which underlie the pathological chronification of pain. These studies allow us to propose that the

persistence of the neuronal excitability of the chronic pain syndromes could be related to dynamic epigenetic changes that modify the phenotype of neuron and glial cells.

DNA methylation, which associates heterochromatin formation and transcription silencing, constitutes a major epigenetic mark in mammals. Recent studies suggest that DNA methylation changes play a crucial role in the process of pain chronification. However, we are still far from understanding how DNA methylation contributes to pain chronification and, specifically, the role played by the crosstalk between miRNAs and this epigenetic mark in the neuropathic pain setting. In this regard, it has been reported that deregulation of miR-30c-5p, under several pathological conditions, brings about changes in the expression and function of DNMTs with pathophysiological implications. In addition, the "de novo" DNMT3b is consistently predicted as a target for miR-30c-5p in the online miRNA bioinformatics tools (TargetScan4.0, miRanda and miRbase).

DNA methylation represses gene transcription through several mechanism including physically blocking the binding of transcription factors or functioning as docking sites for transcriptional repressors. Therefore, it is possible that some epigenetic mechanisms are playing a key role in the pathologic neural plasticity processes that lead to the chronic neuropathic pain state. How DNMTs contribute to neuropathic pain genesis and establishment is still elusive and the possibility to interfere in the painful process through modulation of epigenetic mechanism mediated by miR-30c-5p *in vivo* modulation could suppose the opening of new therapeutic perspectives for the treatment of pathological pain situations highly resistant to conventional pharmacological treatment.

On the other hand, little is known about the changes that occur at a cellular level in the DRG neurons following the neuropathic lesion. DRGs are the first step in the nociceptive pathway and probably the first neurons to be affected. Although structural and functional alterations in some cellular organelles have been described in other neurodegenerative diseases such as lateral amyotrophic sclerosis or spinal muscular atrophy, we have not found any published data that investigate the effect of sciatic nerve injury, which causes neuropathic pain in animal models, at a more cellular level. Understanding

the cellular alterations caused by a sciatic nerve lesion and how miR-30c-5p modulation could induce changes in the organization and functions of the cellular components are essential to understand the pathophysiology and aberrant plasticity mechanism involved in the neuropathic pain disease caused after a nerve damage.

Considering all this data, we hypothesized that epigenetic modifications and cellular alterations are involved in the pathological plasticity of the nervous system that underlies establishment and development of neuropathic pain after a neuronal lesion. Furthermore, miR-30c-5p could be involved in the aberrant epigenetic mechanisms underlying neuropathic pain by modulating the activity of DNA methyltransferases. Based on our hypothesis, we proposed the following general objectives:

I. To stablish the global levels of DNA and histone methylation and its distribution pattern in two pain-related areas, the dorsal root ganglia and the dorsal horn of the spinal cord, in rats subjected to sciatic nerve injury and evaluate the effects of miR-30c-5p-gain- and –loss- of function *in vivo*.
II. Asses the cellular alterations (protein synthesis machinery and nucleolar organization and structure) in neurons of the DRG that occur in rats exposed to sciatic nerve injury and the consequences of miR-30c-5p modulation.

Introduction

1. Pain

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage". Pain, unlike other conditions, is the most important element of interference in the life of the affected person at a biological, psycho-emotional and social level. For this reason, it is considered a key indicator of health, welfare and quality of life. Pain is an indispensable sensation that under physiological conditions warns the body to protect itself from a tissue damage or disease. The painful physiological feeling is proportional to the intensity of the stimulus and triggers protective responses directed to the defense of the organism. The pain usually disappears after the healing of the lesion that caused it. However, in specific conditions of neural damage or inflammation, pathologic plasticity events occur in the nervous system (Woolf and Ma, 2007; Basbaum *et al*., 2009; Li *et al*., 2016; Gwak *et al*., 2017). This leads to abnormal painful sensations in the nociceptive system such as prolonged and persistent pain when the lesion that caused it disappeared or a disproportionate painful response to the causal stimulus. In these situations, pain loses its protective function and it stands as a pathological process that requires specific treatment (Cerveró, 2009). Prolonged suffering caused by chronic pain is one of the main reasons of medical consultation as chronic pain can produce a negative impact on the physical, psycho-emotional, personal, family, work, social and economic level of the affected person and their environment. Chronic pain is considered a pathological process itself, refractory to conventional analgesic drug therapy (Cerveró, 2009).

1.1 The nociceptive system

The nociceptive system is the one responsible for the detection and processing of the noxious stimuli, transforming it into electrical signals, which are then conducted to the central nervous system (CNS). The neurophysiological processes that participate in pain are:

a) *Activation and sensitization of the peripheral nociceptors*

Pain receptors are called nociceptors. A nociceptor is a primary nociceptive-sensory neuron that responds to potential damage stimuli by sending nerve signals to the dorsal horn of the spinal cord. The cellular bodies of the nociceptors are located in the dorsal root ganglion or the trigeminal ganglion (Flórez, 2007; Woolf and Ma, 2007). Nociceptors are able to distinguish between innocuous and noxious stimuli as they are able to encode the intensity of a stimulus between a range of noxious intensities, while they are not able to respond or respond irregularly to low intensity stimuli (Woolf and Ma, 2007). Nociceptors are distributed through the body and are present in many body tissues including the skin, viscera, muscles, joint, meninges and connective tissue. The rest of body tissues have lower levels of nociceptive endings. These receptors transmit the information through nerve fibers that are classified according to the speed of transmission, which is directly correlated with the diameter of axons of the neurons and whether or not they are myelinated. Nociceptive fibers have been classified on the basis of their conduction speed and sensitivity (Meyer *et al*., 2006; Flórez, 2007; Dubin and Patapoutian, 2010) (**Figure 1**):

- Aα/β fibers are highly myelinated and of large diameter axons (6-20 μm), therefore allowing rapid signal conduction (30-120 m/s). They have a low activation threshold and usually respond to light touch and transmit non-noxious stimuli.
- Aδ fibers are lightly myelinated and have medium diameter (1-5 μm), and hence have intermedium conduction speed (12-30 m/s). They respond to mechanical and thermal stimuli, although they can also respond to innocuous stimuli. They carry the first feeling of pain, what is also called "rapid pain" (about 300 ms) and are responsible for the initial adaptive response to acute pain (the withdrawal response). It is a well delimited, localized (epicritic) and sharp pain.
- C fibers are unmyelinated and have small diameter axons (0.3-1.5 μm). Hence, they demonstrate the slowest conduction velocity (0.4-2 m/s). C fibers are polymodal, responding to mechanical, chemical and thermal harmful stimuli although they can also respond to innocuous stimuli. They are

responsible of the transmission of the "second pain"or "slow pain" (approximately 0.7-1. S). It is a bad localized (protopathic) and burning pain.

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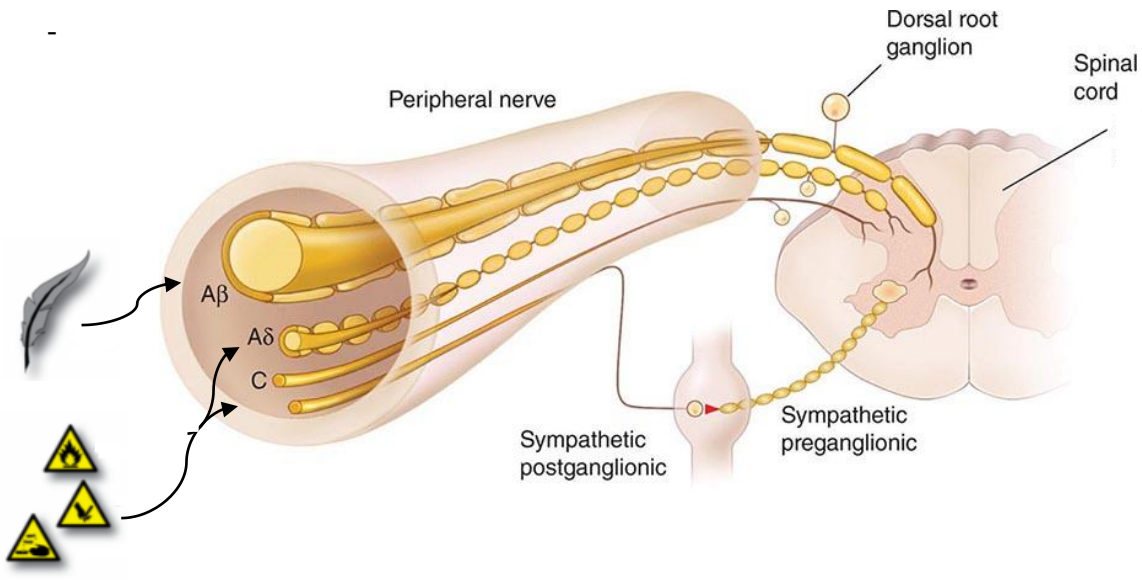


Figure 1: Anatomy of the nociceptors. Types of sensitive primary neurons and degree of myelination; primary afferents include those with large-diameter myelinated (Aβ), small-diameter myelinated (Aδ), and unmyelinated (C) axons (Taken from Rathmell and Fields, 2015).

Nociceptors can be classified according to their localization and the type of the stimulus that they respond to. There are four types of nociceptors:

- *Cutaneous nociceptors*: They present a high stimulation threshold and only become activated in response to intense stimuli. They are inactivated when there are no noxious stimuli. There are two main categories of cutaneous nociceptors:
  - i) Aδ mechanical nociceptors: They are located in the superficial layers of the dermis, with branches that extend to the epidermis and respond to noxious stimuli of mechanical type (sharp pain).
  - ii) C-polymodal nociceptors: They are free endings in the skin and respond well to noxious mechanical, thermal and chemical stimuli. They are very sensitive, leading to the phenomenon called primary hyperalgesia. They are called "polymodal nociceptors" as they can respond to a wild range of noxious stimulus. Nociceptors can also be classified according to the differential expression of channels

that cause heat sensations (TRPV1), (TRPM8) and the ones that are activated by chemicals (TRPA1) (Basbaum *et al*., 2009).

- *Visceral nociceptors*: They innervate internal organs such as the heart, lungs, vascular system, testis, uterus and ureter. Most of the visceral nociceptors are free nerve endings of C, and in some cases, Aδ fibers.
- Joint nociceptors: The joint capsule, ligaments, periosteum and joint fat have C and Aδ fiber endings that respond to low threshold joint movements or noxious ones and to factors released by tissue damage such as inflammation.
- *Muscle nociceptors*: Muscles are innervated by Aδ fibers and respond to allogenic molecules (potassium ions, bradykinin or serotonin) or sustained muscle contractions and C fiber terminations that respond to noxious muscle stimulus such as pressure, heat or muscle ischemia. It is a diffuse pain, difficult to locate, dull and continuous.

- b) *Nociceptive stimuli transmission through the primary afferents*

Tissue pain triggers the production and release of chemical mediators with allogenic properties from the primary sensory terminal and from non-neural cells (for example, fibroblast, mast cells, neutrophils and platelets) in the immediate environment of the peripheral sensory ends or nociceptors (**Figure 2**). Some of these molecules are: ions (H^+ and K^+), bradykinin, prostaglandins, serotonin, noradrenalin, histamine, substance P, thromboxanes, protons, cytokines, tumor necrosis factor (TNF) and neurotrophins (specially the nervous growth factor), are also produced during inflammation. Some of these factors directly activate the nociceptor while other factors act in a synergic way, increasing the response or sensitizing (decrease the threshold) of the nociceptor, which play a crucial role in the primary hyperalgesia processes. Activation of the nociceptor not only transmits afferent messages to the spinal cord dorsal horn (and from there to the brain), but also initiates the process of neurogenic inflammation. These factors also activate many non-neuronal cells, including mast cells and neutrophils which in turn contribute as additional elements to the inflammatory process that are able to stimulate and sensitize peripheral nociceptors and spinal cord pathways of painful transmission (Julius and Basbaum, 2001; Woolf and Ma,2007).

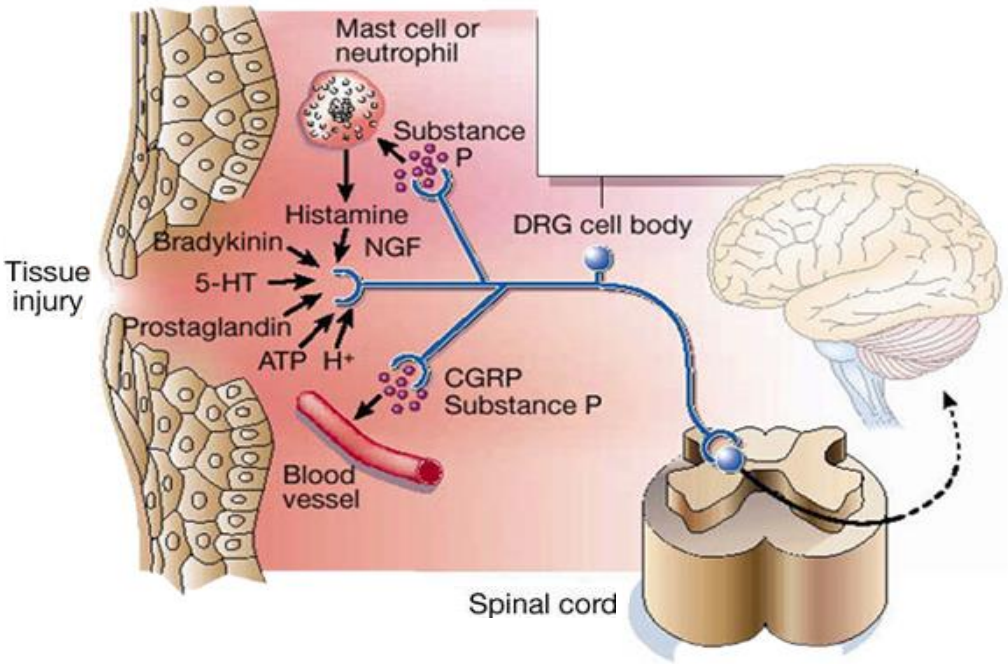


Figure 2: Inflammatory mediators released at the site of tissue injury. Some of the main components that facilitate the inflammatory processes and stimulate pain transmission include peptides (bradykinin), lipids (prostaglandins), neurotransmitters (serotonin (5-HT) and ATP) and neurotrophins (NGF). (Taken from Julius and Basbaum, 2001).

c) Modulation and integration of the nociceptive response at the dorsal horn of the spinal cord level

The axons of primary afferent nociceptors enter the posterolateral sulcus of the spinal cord, ending in the dorsal horn of the spinal cord gray matter. The primary neuron of the pain transmission pathway has an ending in the periphery, the body in the dorsal root ganglia (DRG) and the central ending in the dorsal horn of the spinal cord (SDH). Several excitatory neurotransmitters are released in the transmission of the nociceptive impulse from the periphery to the second neuron in the SDH. Nevertheless, the spinal cord is not only a point in the transmission of nociception, but represents a place of important interactions where a nociceptive impulse is allowed to follow the way towards higher structures or is totally or partially blocked (Meyer *et al*.,2006; Todd and Koerber,2006).

The gray matter of the spinal cord is organized into a series of layers called "Rexed laminae". The central terminals of the primary afferent fibers end in

the gray matter of the dorsal horn of the spinal cord. Most of the primary nociceptive neurons terminate superficially in laminae I, II, IV and V of the dorsal horn (**Figure 3**), where they synapse with the secondary afferent neurons to carry pain information to the brain (Flórez, 2007; D'Mello *et al*., 2008).

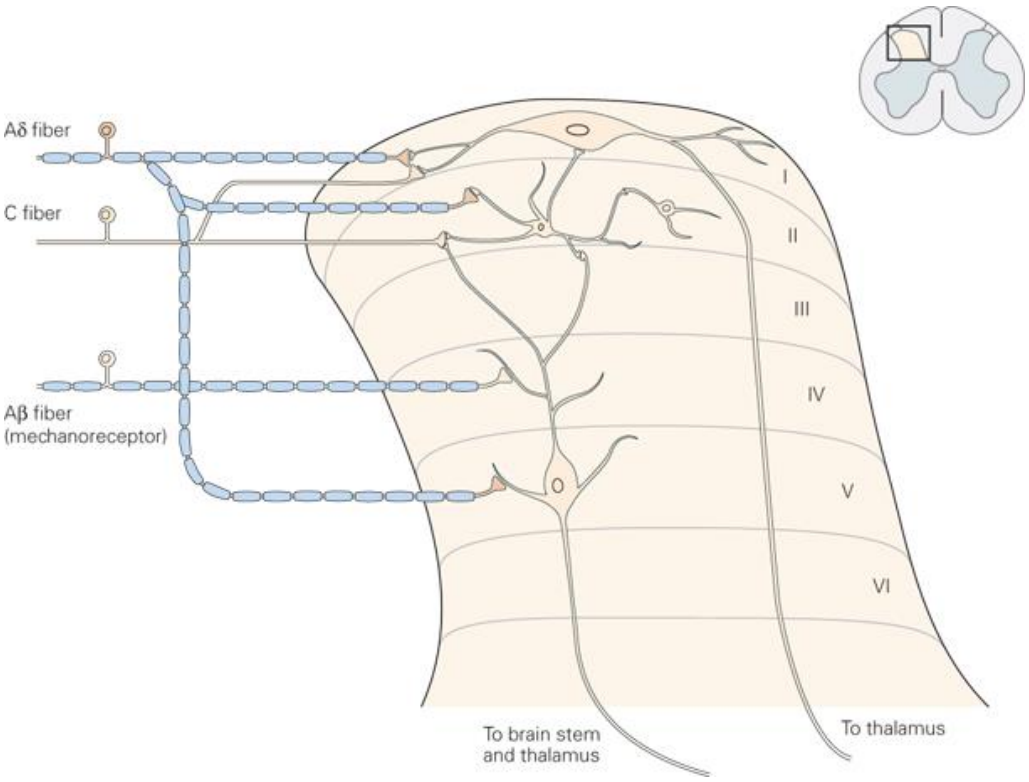


Figure 3: Schematic representation of the Rexed laminae organization in the dorsal horn of the spinal cord and afferent Aβ, Aδ y C fibers. (Taken from Kandel, 2000).

The distribution of the cells and fibers within the gray matter of the spinal cord exhibits a pattern of lamination. Laminae I, also called the marginal zone, contains mainly supraspinal projection neurons that respond exclusively to noxious stimuli, sending direct connections to the thalamus and to the different spinal segments. Lamina II is also called the substantia gelatinosa of Rolando. This layer is composed of tightly packed excitatory (glutamatergic) e inhibitory (glicinergic and GABAergic) interneurons that are essential for nociception processing and respond to noxious stimuli. The majority of the neuron axons in Rexed lamina II receive information from sensory dorsal root ganglion cells as well as from descending dorsolateral fasciculus fibers. They send axons to Rexed laminae III and IV. Lamina IV receives predominantly non-noxious information. Lamina V is composed by

wide dynamic range neurons that are able to identify different pain intensities. Aδ fibers end in the I and V lamina, C fibers in the I, II and V and Aβ in the II, IV and V. Generally, nociceptive afferents end in lamina I and II and V, and the non-nociceptive afferents of low threshold end in the deep II, IV and V layers. Lamina X is also related to the visceral pain nociceptive transmission.

d) *Transmission through the ascending projection pathways*

The ascending spinal cord projections anatomically connect second-order neuron of the spinal cord and the upper nerve centers. They are located in the ventral, lateral and dorsal funiculi on each side of the spinal cord (**Figure 4**) (Bonica, 2001; Dostrovsky and Craig, 2006; Flórez, 2007; García-Porrero and Hurlé, 2014).

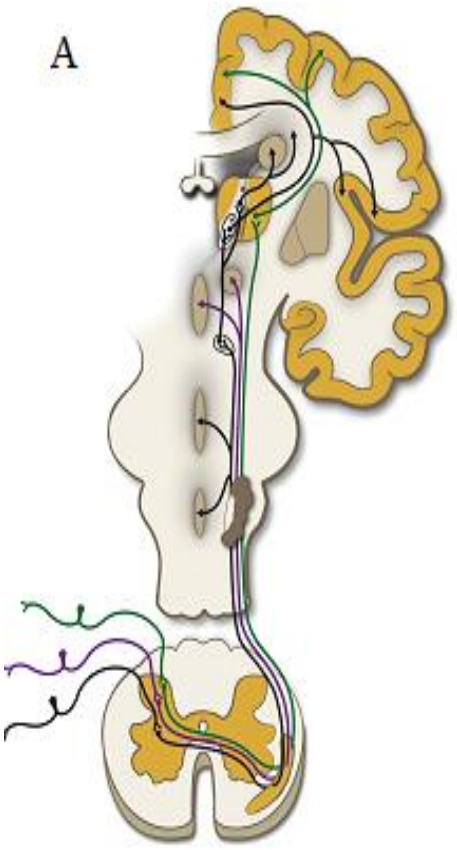


Figure 4: Ascending and descendending pain pathways. A) Anterolateral-spinotalamic somatostetic system. Spinothalamic tract fibers are represented in green; spinoreticular tracts in black; and spinomesencefalic tracts in violet.

The spinothalamic tract, conveys nociception, temperature, non-discriminative touch and pressure information to the somatosensory region of the thalamus. It is composed of a ventral (anterior, paleospinothalamic) and a lateral (neospinothalamic) pathway. 50% of the neurons of this tract are located in the lamina I, but also in the IV-V and V-VIII ones. 90% of the neurons decussate to the contralateral side of the spinal cord, the ones that decusse in the Lamina I form the spinothalamic lateral tract (Aδ y C afferents; thermoanalgesic sensibility) and the ones from the V-VII form the spinothalamic anterior tract (afferents Aβ, Aδ y C; crude touch and pressure sensations). Main projections connect with the third-order neuron in the medial and intralaminar nucleus of the thalamus.

e) *Pain processing in upper centers (encephalic structures)*

The classic pain pathway consists of a three-neuron chain that transmits information from the periphery to the spinal cord and relays the signal to the thalamus before terminating in the cerebral cortex. Ascending projections allow the anatomic connections between the second-order neuron of the spinal cord and the upper nervous centers, so that the perceived intensity of the painful impulses is correlated with an increase in the activity of a great number of brain structures (Apkarian *et al*., 2011; Hayati and Badariah,2014) (**Figure 5**). The main areas of the brain involved in pain perception are:

- *Primary somatosensory cortex* plays an important role in pain perception. It is the main area on which pain is perceived, location and intensity is assessed. Its activation is modulated by cognitive factors such as attention or previous experiences, which alter pain perception.
- The *thalamus* is one of the structures that receives projections from multiple ascending pain pathways. The thalamic nuclei are involved in the sensory discriminative and affective motivational components of pain. The structure is not merely a relay center but is involved in processing nociceptive information before transmitting the information to various parts of the cortex (Melzack and Casey, 1968). Spinal lamina I neurons project extensively to the ventrobasal complex (ventral posterolateral + ventral posteromedial) and to the posterior thalamic nuclei (Dado *et al*., 1993). The nociceptive neurons from the ventrobasal complex mainly

project to the primary somatosensory cortex and this pathway constitutes the lateral pain system that plays an important role in the discrimination of stimuli.

- The *amygdala* integrates the aversive component of the painful experience, such as anxiety, fear avoidance, dangerous or painful situations.
- The nucleus *Raphe magnus* is involved in pain mediation; it sends projections to the dorsal horn of the spinal cord to directly inhibit pain. When stimulated, it releases serotonin, participating in the endogenous analgesia system.
- The *locus coeruleus* is the noradrenergic center and is involved in the descendent control of pain and the emotional component of pain.
- The *periaqueductal gray* (PAG) is the primary control center for descending pain modulation and the defensive behavior. Activation of this area releases encephalin by neurons that project to the nucleus Raphe magnus, producing a release of serotonin that is carried through the descendent pathways producing an inhibitory effect over the entrance of noxious stimuli.
- The *hypothalamus* is responsible for regulating hunger, thirst, response to pain, levels of pleasure, anger and aggressive behavior. Paraventricular and ventrolateral nuclei have neuroendocrine functions, such as pain arousal, thermoregulation, changes in blood pressure and other homeostatic function.

1.1.1 Pain modulation circuits

Contrary to the nociceptive ascending centripetal transmission, endogenous inhibitory system is descending and centrifugal. There are mechanisms that act to inhibit pain transmission at the spinal cord level and via descending inhibition from upper centers. Pain descending modulating systems exert an inhibitory and activator action over the nociceptive afferent, using some molecules such as noradrenaline, serotonin, opioids and cannabinoids (Ossipov *et al*., 2010). Specifically, descending systems are originated in the periaqueductal grey (PAG), the rostral ventromedial medulla (RVM), including

the nucleus Raphe magnus, amygdala, the paragigantocellular reticular nucleus and neurons of the adjacent reticular formation.

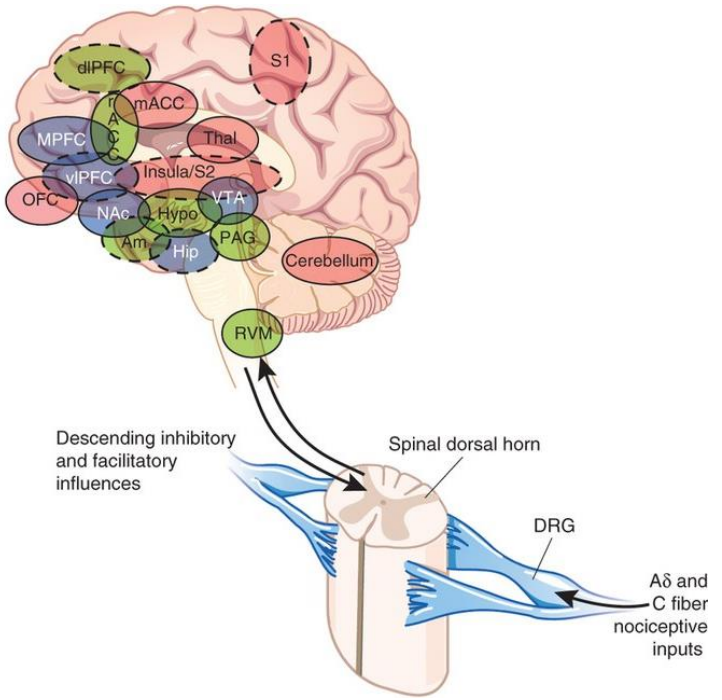


Figure 5: **Ascending pathways, subcortical and cortical structures related with pain processing.** PAG, periaqueductal gray; PB, prabraquial nuclei; VMpo, núcleo ventromedial del tálamo; MDvc: núcleo dorso medial del tálamo; VPL, núcleo lateral ventro-posterior del tálamo; ACC, corteza cingular anterior; PCC, corteza cingular posterior; HT, hipotálamo; S-1 y S-2, áreas corticales somatosensoriales primera y segunda; PPC, complejo parietal posterior; SMA, área motora suplementaria; AMYG, amígdala; PF, corteza prefrontal. (Taken from Price, 2000).

The PAG in the midbrain and the rostral ventromedial medulla are two important areas of the brain involved in descending inhibitory modulation. PAG receives afferents from the prefrontal limbic cortex, amygdala, hypothalamus and spinal neurons. Thus, it receives information about the emotional and motivational state of the person and information regarding the somatic afferents. The balance of this activity over the PAG will allow to regulate pain sensibility. When stimulate, PAG activates encephalin-releasing neurons that projects to the Raphe nuclei. This last structure produces serotonin (5-HT) that descends to the dorsal horn of the spinal cord where it forms excitatory connections with the inhibitory interneurons of the laminae II. When activated, these interneurons release encephalin or dynorphin

(endogenous opioid neurotransmitters) which bind to mu opioid receptors on the axons of incoming fibers carrying pain signals. Two types of neuronal system have been described in the Raphe magnus nucleus: the "on" cell system that increases the activity after the nociceptive stimulus, remaining the whole time that the motor response lasts and favors pain transmission; and the "off" cell system that exerts an inhibitory influence over that transmission. Both systems influence each other and their activity is alternating, so when some are activated, the others are inhibited.

"Off" cells are characterized by interrupting their activity before the reflex response occurs and their activity is promoted by endogenous and exogenous opioids (Bonica, 2001; Flórez, 2007; Ossipov *et al*., 2010).

One of the mechanisms consists in the activation of inhibitory interneurons present in the spinal cord when they contact with dendrites or bodies of the ascending projecting neurons carrying cutaneous sensory. Activation of inhibitory interneurons inhibits and modulates pain transmission information carried by the pain fibers. This presynaptic inhibition system affects all primary afferent fibers and acts as an auto-control mechanism of afferent impulses of complementary sensory fibers (Aliaga *et al*., 2009).

1.2 Types of pain

1.2.1 Nociceptive pain

Also known as physiologic pain, arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors. It is divided into somatic or visceral pain. Somatic pain affects the skin, muscles, joints, articulations or bones (well localized and limited to the injured area). Visceral pain affects organs (bad localized, referred and accompanied with vegetative responses) (Bonica, 2001). Inflammatory nociceptive pain is triggered by tissue breakdown, high pressures, burns, intense or prolonged cold, chemical lesions that arise an inflammatory response that releases a huge amount of molecules that stimulate nociceptors.

It is an acute, sharp, aching, throbbing, dull, of a medium-high intensity, short duration pain that usually can be controlled if the cause of the irritation ends and its function is to alert the body from intense thermal, mechanical and chemical noxious stimuli (endogenous and exogenous). Nociceptive pain

can be temporal, but sometimes, depending on the pathology can generate recurrent stimuli producing chronic nociceptive pain. This type of pain activates C and Aδ fibers and is mostly mediated by TRPV channels (Basbaum *et al*., 2009).

1.2.2 Neuropathic pain

According to the IASP, neuropathic pain is a type of pain that arises as a direct consequence of a lesion or disease of the somatosensory nervous system (IASP, 2011). Neuropathic pain can be classified according to the location of the neuronal damage in:

- Peripheral neuropathic pain affects the peripheral nervous system (PNS). It originates from a damage to the peripheral nerve, plexus, dorsal root ganglion or root. Diabetes is the most common cause of this type of neuropathy, although it can also be caused by traumatic or herpetic injuries, autoimmunity disorders, chronic renal disease, metabolopaties, prolonged exposure to extreme cold, direct pressure in a nerve, etc.
- Central neuropathic pain affects the central somatosensory system, originating from a damage of the brain or spinal cord. The most frequent causes of central neuropathic pain are medullar lesions, brain ischemia, multiple sclerosis, etc.
- Mixed neuropathic pain which affects the CNS and PNS.

Neuropathic pain is a type of chronic pain caused by the injury or illness of the somatosensory nervous system. Some individuals develop a persistent pain after the healing of a nerve injury. This pain is characterized by exaggerated responses to painful stimulus (hyperalgesia), pain in response to harmless stimulus (allodynia) and spontaneous pain which can remain for months or years after the healing time (Cerveró, 2009). Often, patients with neuropathic pain do not response to currently available treatments (Finerup *et al*., 2015; Colloca *et al*., 2017). Neuropathic pain is a very prevalent pathological process, which affects 3-10% of the general population and constitutes the most frequent cause of demand for medical care. Secondary neuropathy after chronic ischemia of the lower extremities, the phantom limb syndrome after amputation, tumor processes, diabetic neuropathy, postherpetic neuralgia, fibromyalgia, etc are frequent causes of neuropathic pain (Hsu and Cohen, 2013; van Hecke *et al*.,2014).

Pain chronification after a neural lesion is a consequence of pathological plasticity process that is established in structures of the CNS and PNS responsible for reception, processing and modulation of nociceptive sensitivity (Basbaum *et al*., 2009; Li *et al*., 2016; Gwak *et al*., 2017). The final result of these pathological adaptations is a neuronal hyperexcitability of very long duration in front of noxious or even harmless stimulus. The hyperreactivity of the PNS and CNS causes sub-threshold stimulus to be able to evoke electrical response in the specific pathways. In these situations, peripheral stimulation causes hyperalgesia and hyperesthesia (exaggerated reactions to physiological responses), allodynia (painful perception to non-harmful thermal or tactile stimulus), hyperpathy (exaggerated response to repetitive stimulus) and dysesthesia (abnormal perception of daily stimulus). Spontaneous pain can also appear without apparent stimulus, occupying a peripheral nervous territory.

Although the molecular and cellular mechanism that contribute to the phenomenon of sensitization have been exhaustively studied, we still do not know what are the elements that determine the persistence of pain after the healing of neural damage and what factors condition the individual susceptibility to suffer this type of pathology.

Peripheral sensitization (primary hyperalgesia): The first element in the course of neuronal sensitization is the inflammatory process in the area of the injury. Tissue damage causes the release of numerous mediators from the local primary nociceptive neurons, cells resident in the damaged area and from inflammatory infiltrated cells from the bloodstream (Basbaum *et al*., 2009; Echeverry *et al*., 2013). Ectopic (outside the habitual place where they should be generated) potential actions are generated within the signaling nociceptive channels. This ectopic environment propitiates that axons transmit continuously and without the need of a stimuli an action potential that is interpreted as a pain feeling in the upper brain areas. The different elements of the so-called "inflammatory soup" (H^+, proteases, adenosine, bradykinin, interleukin, adenosine triphosphate (ATP), histamine, prostaglandins, neurotrophins, factor tumor necrosis (TNF), substance P, peptide related to the calcitonin gene (CGRP), neuroregulin, chemokines, etc) (i) directly excite the membrane of the nociceptor, activating it; (ii) cause

sustained changes in gene expression (ionic channels, receptors coupled to G proteins, receptors with tyrosine kinase activity, etc) in the primary nociceptive neurons of the DRG and in the satellite glia, which lead to their hypersensitivity; and (iii) they recruit new inflammatory elements to the injured area (Basbaum *et al*.,2009). There is also a decrease in the activation threshold of many ionic channels of the nociceptors, specially TRPV1 and vaniloid receptor that plays a crucial role in the peripheral sensitization, allowing the entrance of Na^{+} and Ca^{+2}, in the damaged area an in the proximal area of the injury (Davis *et al*., 2000: O'Neill *et al*., 2012). All this leads to a reduction of the nociceptive threshold and consequent hypersensitivity to thermal stimuli and mechanical (peripheral sensitization). All of this results in pain hypersensitivity symptoms confined to the site of the inflamed tissue. This is referred to as the zone of primary hyperalgesia.

*Central sensitization (secondary hyperalgesia): C*hronic neuropathic pain often extends spatially beyond the area of the initially involved root or nerve to create a zone of secondary hyperalgesia, which often becomes independent of the initial noxious event. These symptoms cannot be explained by changes in the PNS, but rather reflect changes in spinal and supraspinal networks that culminate in a functional shift of the sensory system from physiological high-threshold nociception to pathological low-threshold pain hypersensitivity (Ikoma *et al*.,2003). The sustained activity of peripheral terminations of the nociceptor promotes a profuse release of amino acids and excitatory neuropeptides (P substance, glutamate and nitric oxide) to the second-order neurons of the dorsal horn of the spinal cord, leading to postsynaptic changes, as well as the phosphorylation of NMDA and AMPA receptors and to an overexpression of Na^{+} voltage dependent channels. At this level, activated immunocompetent glial cells and infiltrated lymphocytes release soluble mediators that diffuse to neighboring zones modulating presynaptic and postsynaptically neuronal excitability (Salter and Beggs, 2014). The interactions between primary afferents, second-order neurons, activated spinal glia and infiltrates inflammatory (Grace *et al*., 2014) lead to central sensitization, characterized by: (i) threshold of response to reduced afferent stimuli; (ii) hyperexcitability and activity neuronal spontaneous; (ii)

expansion of connectivity and synaptic area of influence; (iii) transformation of non-nociceptive neurons into nociceptives.

Mechanisms similar to these operate not only in the spinal cord, but also in supraspinal levels such as the somatosensory, anterior cingulate, prefrontal and insular cortex, amygdala or gray matter periaqueductal (Baron *et al.,* 2010). They have been described even in brain areas not directly associated with the processing of sensory information. This aberrant plasticity results in the apparition of pain in response to innocuous not-painful stimulus (allodynia) and exaggerated pain after a stimulus that in normal conditions is painful, which becomes much more painful (hyperalgesia) in the area of the lesion as well as in remote spraspinal areas, which may persist for very long periods of time (**Figure 6**) (Campbell *et al*., 2006; Latremoliere and Woolf, 2009).

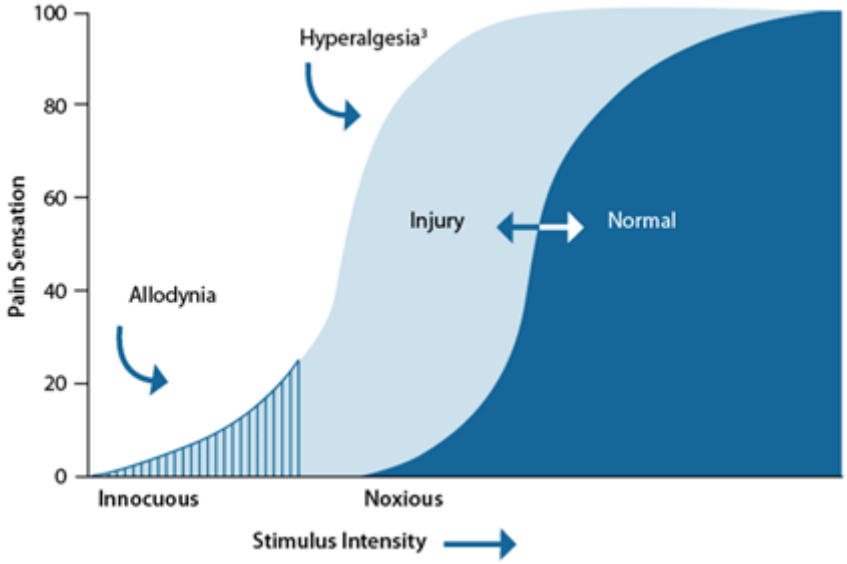


Figure 6: **Representation of the difference between allodynia and hyperalgesia.** In allodynia, an innocuous stimulus is perceived as painful. With hyperalgesia, there is an increased sensitivity to injury (Taken from Medscape).

Loss inhibitory systems: Inhibitory interneurons of the spinal cord are responsible for restringing the development of hyperalgesia and allodynia after neural damage. In neuropathic pain, there is a loss in the number of these inhibitory interneurons. In the remaining neurons there is a decrease in the expression of inhibitory receptors (in the primary afferents and in postsynaptic neurons) after the nerve injury that contributes to the

sensitization and exacerbation of pain due to a reduction in the transmission of the supraspinal inhibitory signals (Kohno *et al*., 2005). The dorsal horn of the spinal cord receives afferent fibers from the supraspinal centers that have a modulator function in the descendent control of pain: the loss of descendent inhibitory signals (opioid, serotonergic and noradrenergic) that are originated mainly in the PAG and in the locus coeruleus, contributes to hyperalgesia and central sensitization as well as pain chronification (Fields *et al*., 2006). With the loss of neuronal input (deafferentation) the spinothalamic tract neurons begin to fire spontaneously, a phenomenon designated "deafferentation hypersensitivity" (Hanakawa, 2012).

Not only neurons are involved in neuropathic pain but also Schwann cells, satellite cells of the dorsal root ganglia, microglia, astrocytes and components of the immune system (P substance, bradykinin, PRGC, NO, macrophages, lymphocytes T, cytokines, etc.) (Scholz *et al*., 2007; Austin and Mohalem-Taylor, 2010). Microglia constitutes one important source of inflammatory mediators, thus inducing the release of allogenic molecules and respond to pro-inflammatory signals released by other non-neuronal cells, mainly immune cells (Grace *et al.*,2011; Mika *et al*.,2013).

Following peripheral injury, microglia proliferate, become hypertrophic and activated and secrete molecules which sensitize sensory neurons in the spinal cord. The best-characterized mechanism involves the activation of microglial purinergic receptors P2X4 by ATP secreted from damaged neurons, astrocytes or both. This stimulates microglia to release brain-derived neurotrophic factor (BDNF), which activates its neuronal receptors (Tsuda, 2016; Malcangio, 2016). Glia have emerged as key contributors to pathological and chronic pain mechanisms. Upon activation, these mediators cause an inflammatory reaction, hyperemia and chemotaxis, contributing to the nociceptive sensibility and inducing the appearance of hyperalgesia and allodynia (Costigan *et al*., 2009; Chiu *et al*., 2012).

The intercommunication neuron-astrocyte-microglia after the injury of the CNS seems to be based on an exchange of molecules such as cytokines (Milligan and Watkins, 2009, Baron *et al*., 2010; Calvo *et al*., 2012; Skaper *et al*., 2012; Taves *et al*., 2013). Transforming growth factor-β (TGF- β) constitutes a family of pleiotropic, contextually acting cytokines (Massagué,

2012). Emergent evidence supports a protective role for TFG-β signalings againts the pathological neural plasticity underlying neuropathic pain in animal models (Echeverry *et al*., 2009; Tramullas *et al*., 2010; Lantero *et al*., 2012; Echeverry *et al*., 2013). Recently is has been discovered the existence of crossed interactions between TGF-β signaling and some microRNAs, through feedback circuits (Butz *et al*., 2012). Most of the elements of the TGF-β signaling pathway are regulated by miRNAs and, at the same time, TGF-β signaling increases the biogenesis of a subgroup of miRNAs, leading to a bidirectional interaction. Some of the miRNAs interrelated to TGF-β have been related to the aberrant neuronal plasticity that underlies the development of tolerance to opioid analgesia (Rodríguez, 2012). Previous results from our group also evidence that the interaction between miR-30c-5p and its target TGF-b modulated the endogenous opioid system (Tramullas *et al*., 2018). Therefore, we postulate that therapies focused on modulating the crosstalk between TGF-β signaling and miRNAs could constitute an alternative strategy for chronic pain treatment.

2. Epigenetics

2.1 Chromatin structural organization

Genome of eukaryotic organisms face the enormous challenge of packing an incredibly long linear molecule of DNA into a restricted nuclear volume. To solve this problem, DNA is tightly packaged and associated with basic proteins called histones, forming the chromatin. The basic repeat element of the chromatin is the nucleosome, which consist of a histone octamer and 146 base pairs of DNA wrapped 1.7 turn tightly around it. This histone octamer consists of a central $(H3/H4)_2$ tetramer flanked on either side by two H2A/H2B dimers. Each nucleosome is separated from the next by linker DNA (10-80 base pair long), associated with histone H1 (Felsenfeld and Groudine, 2003). This DNA-nucleosome complex forms a fiber of 11 nm in diameter known as "beads on a string" (Olins and Olins, 1974; Woodcock, 1973). These fibers are then coiled to a helical structure known as the 30 nm fiber, which in turn is condensed to form chromosomes which are visible through light microscope in dividing cells (**Figure 7**)(van Holde and Zlatanova, 1995).

The structure of the chromatin is highly dynamic and it can switch between the heterochromatin (condensed) and the euchromatin (relaxed) form. This flexible structure allows the chromatin to function properly in the cell to package DNA into the nucleus, to strengthen the DNA during mitosis and meiosis and to control gene expression, DNA replication and DNA repair (Felsenfeld and Groudine, 2003). To achieve this high level of coordination in the nuclear processes, cells have developed several mechanisms to spatially and temporally modulate chromatin structure and function on specific loci in the genome. These mechanisms involve chromatin remodeling, incorporation of histone variants and covalent modifications of histones. The "histone tails" (the amino terminal ends of histones) are extended outside the nucleosome core. Thus, they are accessible to enzymes for chemical modifications which in turn affect the histone-DNA interaction and modulate chromatin structure. Several different types of histone modifications are known, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and decimation. The combination of these modifications would produce over a million different possibilities for each nucleosome (Bhaumik *et al*., 2007; Turner, 2007). Unsurprisingly, this astounding condensation of the genome represents a sizeable obstacle to DNA-templated processes such as transcription, replication, and DNA repair. Eukaryotic genomes have dealt with this problem by dynamically manipulating chromatin structure in order to expose underlying DNA sequences. Since histones are intimately associated with DNA, they play an important role in this process.

2.1.1 Types of chromatin

Euchromatin: For transcription to be possible, chromatin must have an open conformation to facilitate the access to the transcription machinery. This state of "accessible" chromatin is called euchromatin and it is visualized as pale regions of the nucleus, in the optical microscope images, or as scattered chromatin domains with electron microscopy. In neurons, euchromatin represents a large proportion of the genome. This is because the neurons are cells that express a great number of genes that are "ready" to be quickly activated, as is the case of proto-oncogenes (Nagel *et al*., 2016).

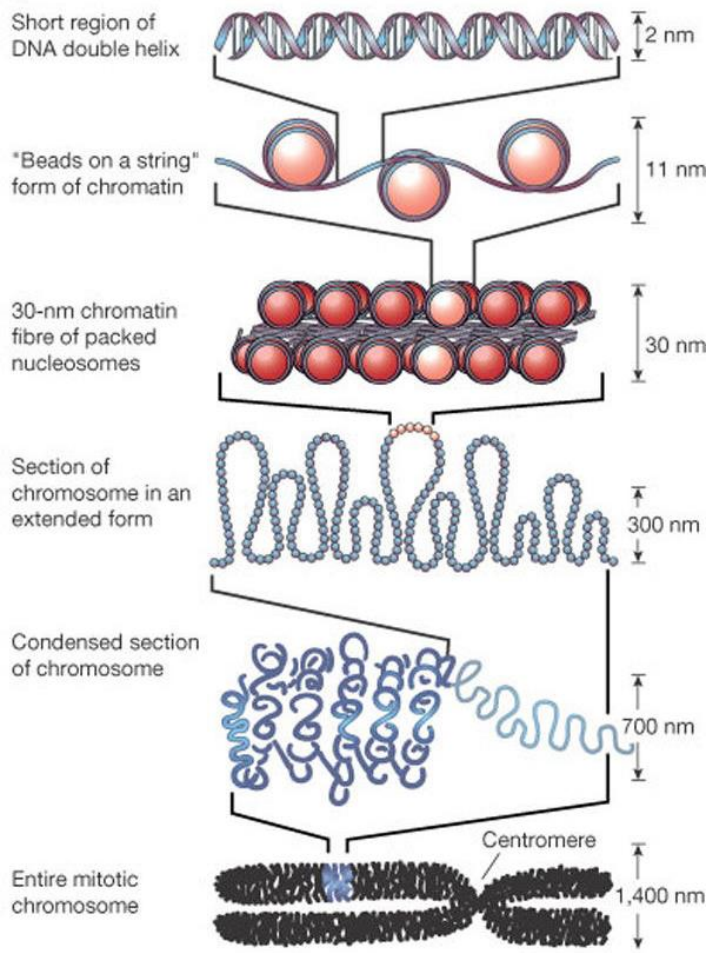


Figure 7: The organization of DNA within the chromatin structure. The lowest level of organization is the nucleosome, in which two superhelical turns of DNA (a total of 165 base pairs) are wound around the outside of a histone octamer. Nucleosomes are connected to one another by short stretches of linker DNA. At the next level of organization, the string of nucleosomes is folded into a fiber of about 30 nm in diameter, and these fibers are then folded into higher-order structures (Taken from Alberts *et al*., 2002).

In the microenvironment of the euchromatin, DNA is very dynamic and can be "unrolled" so that it can be accessed by the transcription, replication or repair machinery of transcription. A determining factor in the configuration of the euchromatin is the histones post-transcriptional modification pattern, "the histone code" characterized by high levels of acetylation of nucleosomal histones as well as by the trymethylation in H3K4, H3K36 and K3K79. This open conformation of chromatin not only facilitates the access of the molecular machinery that carries out the different cellular processes, but also

increases the vulnerability to DNA damage, given its greater exposure to all types of genotoxic agents (Kouzarides, 2007; Wagner *et al*.,2012; Mahrez *et al*.,2016; Hocher *et al*.,2018).

Heterochromatin: The chromatin organization, in two main functional states, was firstly described by Heitz in 1928. While euchromatin constitutes an open and accessible conformation, heterochromatin is highly condensed and transcriptionally inactive. One important function of heterochromatin is to protect the DNA from the transcriptional machinery. Furthermore, heterochromatin is divided into facultative and constitutive. Facultative heterochromatin contains the genes that encodes proteins but should remain silenced during cell differentiation. On the other hand, constitutive heterochromatin corresponds to areas of the genome that are permanently silenced and generally lacks genes that encode proteins. In most of the organisms, constitutive heterochromatin occupies a delimited volume that is grouped in the pericentromeric and telomeric regions. These areas, poor in genes are formed by tandem repeat sequences, also called satellite, that can range from 5 bp to hundreds (Eymery *et al*.,2009; Sullivan *et al*.,2017; Tosolini *et al*.,2018).

The heterochromatin is characterized by some post-translational modifications in the histones that conform the nucleosomes. The most frequent mark in heterochromatin histones is the global hypoacetylation, which contributes to the chromatin packaging. Additionally, the heterochromatin is enriched in specific methylation marks. One of the main mark of constitutive heterochromatin is the trymethylation of the histone H3 in the lysine 9 (H3K9me3) while facultative heterochromatic is marked by the trymethylation of the same histone but in the lysine 27 instead on the lysine 9 (H3K27me) and by the trymethylation of histone H4 on lysine 20 (H4K20me3) (Eymery *et al*., 2009; Shirai *et al*., 2017; Tosolini *et al*., 2018; Zhang *et al*., 2018). Despite having different trymethylation marks, the global result is the same, the chromatin fiber packaging.

2.2 Epigenetics, an overview

Complex organism need epigenetics. While the genetic information in all the cells of an organism is identical, cellular identity and function are highly

diverse. This means that some specialized genes that determine the phenotype of differentiated cells are permanently turned on, and other genes, active in some other cell types, are permanently turned off. Moreover, this cellular identity is mitotically stable, and even when differentiated cells such as fibroblasts or lymphocytes are extracted from their endogenous environment and maintained in the artificial medium of cell culture, maintain their phenotypes through cell division. These terminally differentiated cells are derived from undifferentiated precursors called stem cells. Here an undifferentiated cell divides to produce a differentiated cell, and another undifferentiated stem cell. In the case of bone marrow stem cells, a variety of blood cell types are produced. Thus in addition to its relative stability in differentiated cells, genomic output must be highly malleable during the process of cellular differentiation. This astounding regulatory feat is achieved by a wide range of phenomena that can be collectively grouped under the definition of 'epigenetic', a term initially coined by Conrad Waddington in 1942 to entail, "*processes by which genotype gives rise to phenotype"* (Waddington, 2012).

The word "epigenetics" means "in addition to changes in the genetic sequence" and refers to those heritable changes in gene expression that occur without changes in the DNA sequence (Waddington, 1942). This term has evolved to include all those processes that alter gene activity without changing the DNA sequence and lead to modifications that can be transmitted to the daughter cells (Wu *et al*., 2001). All cells of multicellular complex organisms contain the same genetic information but during development, each single cell differentiates into a specific phenotype without any changes in the DNA sequence. This feature implies that the accuracy of epigenetics modifications is crucial for maintaining the genome integrity and the cell phenotype. Aberrant epigenetic modifications are associated with different heritable and non-heritable diseases. Indeed, epigenetics contributes to the understanding of mechanisms underlying different diseases for which genetic mutations are not the only cause. Epigenetic marks include a variety of gene regulatory events, such as chromatin structure remodeling, histone modifications, DNA methylation and small noncoding RNA, that do not entail changes in the DNA sequence. Therefore, epigenetic events regulate gene

expression at both transcription (histone modification and DNA methylation) and translation (small noncoding RNA) levels.

2.2.1 DNA methylation

DNA methylation is an epigenetic modification essential for maintaining genomic stability, specifying cell fate, genomic imprinting (Li *et al*., 1993), X-chromosome inactivation and stabilization (Heard *et al*., 1997; Sado *et al*., 2000), protection against retroviruses and transposons (Walsh *et al*., 1998) and gene expression regulation (Bird, 2002).

Cytosine nucleotides can be methylated in the 5' position on their pyrimidine ring. Cytosine nucleotides which directly precede guanine nucleotides are known as CpG dinucleotides (Bird, 1986) (where 'p' represents the phosphate bond between cytosine and guanine). DNA methylation occurs almost exclusively in the symmetrical CG context and affects approximately 80% of all CpGs (Ehrlich *et al*., 1982). DNA methylation is also found at sites other than CpGs sequences. This type of methylation is referred to as non-CpG methylation and includes methylation at cytosines followed by adenine, thymine or another cytosine. Non-CpG methylation is nearly absent in adult somatic cells and comprises only 0.02% of total methyl-cytosine in differentiated somatic cells (Lister *et al*.,2009; Laurent *et al*.,2010). Non-CpG methylation seems to have different functions in mouse and human brain tissue. Specifically, it is likely to be correlated with gene activity in human brain tissue, but is negatively correlated with gene activation in the mouse frontal cortex (Lister *et al*., 2009). Recent studies have, however, shown that non-CpG methylation is high in pluripotent stem cells and several other cell types (such as oocytes) and is important for gene regulation (Sharma *et al*., 2015; Patil *et al*., 2016). More recently, it has been discovered that non-CpG methylation plays a role in cardiac gene programing during development (Zhang *et al*., 2016).

The enzymes responsible for DNA methylation are the DNA methyl transferases (DNMTs). These enzymes catalyze the transfer of the methyl group from S-adenosyl-L-methionine (SAM) to the 5' position of the cytosine ring (**Figure 8**). DNMT1, 2, 3A, 3B and 3L are the main DNMTs that belong

to the DNMT family, although only DNMT1, DNMT3A and DNMT3B have methyltransferase activity.

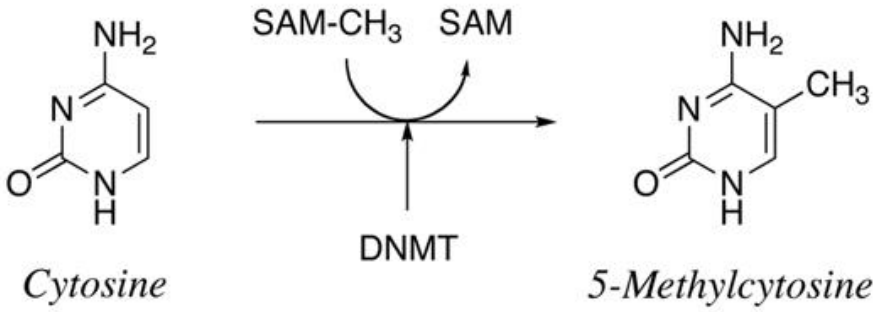


Figure 8: DNA methylation at cytosine residues Cytosine bases (usually within the context of CpG dinucleotides) can be covalently modified by the attachment of a methyl group to the carbon 5 position of the pyrimidine ring in a reaction involving a S-adenosyl-Lmethionine (SAM) methyl donor and the catalytic activity of a DNA methyltransferase (DNMT).

The three DNMTs present in mammals (DNMT1, DNMT3A and DNMT3B) are encoded by separate genes (Chen and Li, 2006) and are considered essential for normal embryonic development (Okano *et al*., 1999). DNMT1 is considered the primary maintenance DNA methyltransferase, methylating newly synthesized DNA during the S phase of the cell cycle (Bird, 2007). DNMT3A and DNMT3B are considered *de novo* DNMTs that are essential for the establishment of DNA methylation patterns during mammalian development and in germ lines (Okano *et al*., 1999). In addition to DNMT3A and DNMT3B, the DNMT3 family include one regulatory factor, DNMT3-Like protein (DNMT3L) that serves as an important cofactor for DNMT3A and DNMT3B but is catalytically inactive due to the mutation of the specific catalytic residues (Bestor, 2000; Pacaud *et al*.,2014).

2.2.1.1 Role and location of DNA methylation

The CpG dinucleotides tend to be concentrated in the promoter region of approximately 60% of the coding genes in humans. An increase in the levels of DNA methylation in the promotor region of the genes is associated with heterochromatin formation and transcription silencing (Keshet *et al*., 1986; Reik *et al*., 2001). Methylated cytosine, also called 5-methylcytosine (5'-meC)

is found in approximately 1.5% of the genomic DNA (Lister *et al*.,2009) and acts as a ligand for methyl-binding proteins (MBPs) that exclusively bind to methylated CpGs (mCpGs) (Parry and Clarke, 2011). The binding of MBPs to mCpGs causes the recruitment of transcriptional repressors, HDACs and chromatin-modifying complexes which in turn induce the formation of heterochromatin, which is the tightly packed and inactive form of the chromatin. These mechanism cause transcription repression, resulting in gene silencing (**Figure 9**) (Nan *et al*., 1998; Fuks, 2005). Methylation can also occur within the gene body, where it is believed to block aberrant transcription and to avoid the production of a truncated form of the protein. Nevertheless, multiple studies have also shown that for some genes, intragenic methylation correlates with an increase in the transcription (Rauch *et al*., 2009; Zilberman, 2017).

Although DNA methylation is relatively stable, DNA demethylation has been observed in a number of different biological processes (Kohli and Zhang, 2013), being essential for maintaining the balance of the DNA methylation levels throughout the genome. DNA demethylation can be active or passive. Passive DNA demethylation requires DNA replication and occurs in the absence of the required DNA methylation machinery (DNMTs or SAM) (Kohli and Zhang, 2013).

On the other hand, active demethylation does not require DNA replication to remove 5'-meC. Instead, it involves the ten-eleven translocation (TET) family of enzymes which oxidize 5'-meC to 5-hydroxymethyl cytosine (5'-hmC) (Tahiliani *et al*., 2009; Ito *et al*., 2010). 5-hmC can be further oxidized to form 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). These oxidized bases are removed by thymine DNA glycosylase-triggered base excision repair (TDG-BER) to reform unmethylated cytosine (He *et al*., 2011; Kohli and Zhang, 2013).

2.2.1.2 DNA methylation in disease

DNA methylation is essential for embryonic development. Absence of DNA methylation in mammals is embryonic lethal, highlighting its biological importance (Auclair *et al*., 2014; Messerschmidt *et al*., 2014; Madakashira *et al*., 2017). Increasing evidence demonstrates that DNA methylation has an

important regulatory role in a great number of diseases. Aberrant DNA methylation is associated with different cancers including ovarian (Ahluwalia *et al*., 2001), breast (Bermejo *et al*., 2018), lung (Esteller *et al*., 2001; Selamat *et al*., 2011), colon (Callie *et al*., 2015; Somasundaram *et al*., 2018; Dong *et al*., 2018) and prostate (Lee *et al*., 2017; Larsen *et al*., 2018). Altered DNA methylation has also been described in neurodegenerative diseases including Alzheimer's disease (Siegmund *et al*.,2007; Sung *et al*.,2011; Roubroeks *et al*.,2017), Parkinson's disease (Matsumoto *et al*.,2010; Chuang *et al*.,2017; Chen *et al*.,2017), amyotrophic lateral sclerosis (Coppede *et al*.,2018; Masala *et al*.,2018), Huntington's disease (Thomas *et al*.,2013; Zadel *et al*.,2018) and autoimmune diseases such as systemic lupus erythematosus (Yeung *et al*.,2017) and multiple sclerosis (Mastronardi *et al*.,2007; Rhead *et al*.,2018). In addition, metabolic diseases such as hyperglycemia (which can lead to type II diabetes) and hyperlipidemia show aberrant DNA methylation (Dayeh *et al*., 2014; Bansal *et al*., 2017). Aberrant DNA methylation levels have also been linked to ischemic heart diseases (Watson *et al*., 2014; Chen *et al*., 2016). In addition, mutations in the DNMT3B gene cause a rare autosomal disease called immunodeficiency centromere instability, facial abnormalities syndrome (Okano *et al.,* 1999; Walton *et al*.,2014; Gatto *et al*.,2017).

With the abundance of emerging evidence indicating the important role of DNA methylation in common diseases, researchers have attempted to use DNA methylation as a biomarker to identify epigenetic changes that are associated with the status of the disease (Dong *et al*., 2018).

2.2.1.3 DNA methylation in pain

Accumulating evidence suggests that epigenetic alterations lie behind the induction and maintenance of neuropathic pain. In fact, peripheral inflammation and nerve injury induce changes in DNA methylation, histone modifications and non-coding RNAs in pain-related regions (Rahn *et al*., 2013; Wang *et al*., 2014; Mauck *et al*., 2014). These changes are thought to be responsible for nerve-injury alterations in some pain-associated genes in central neurons. Epigenetic mechanisms are capable to sustain the long-lasting effects on gene activity in response to environmental stimuli, observed in neuropathic pain.

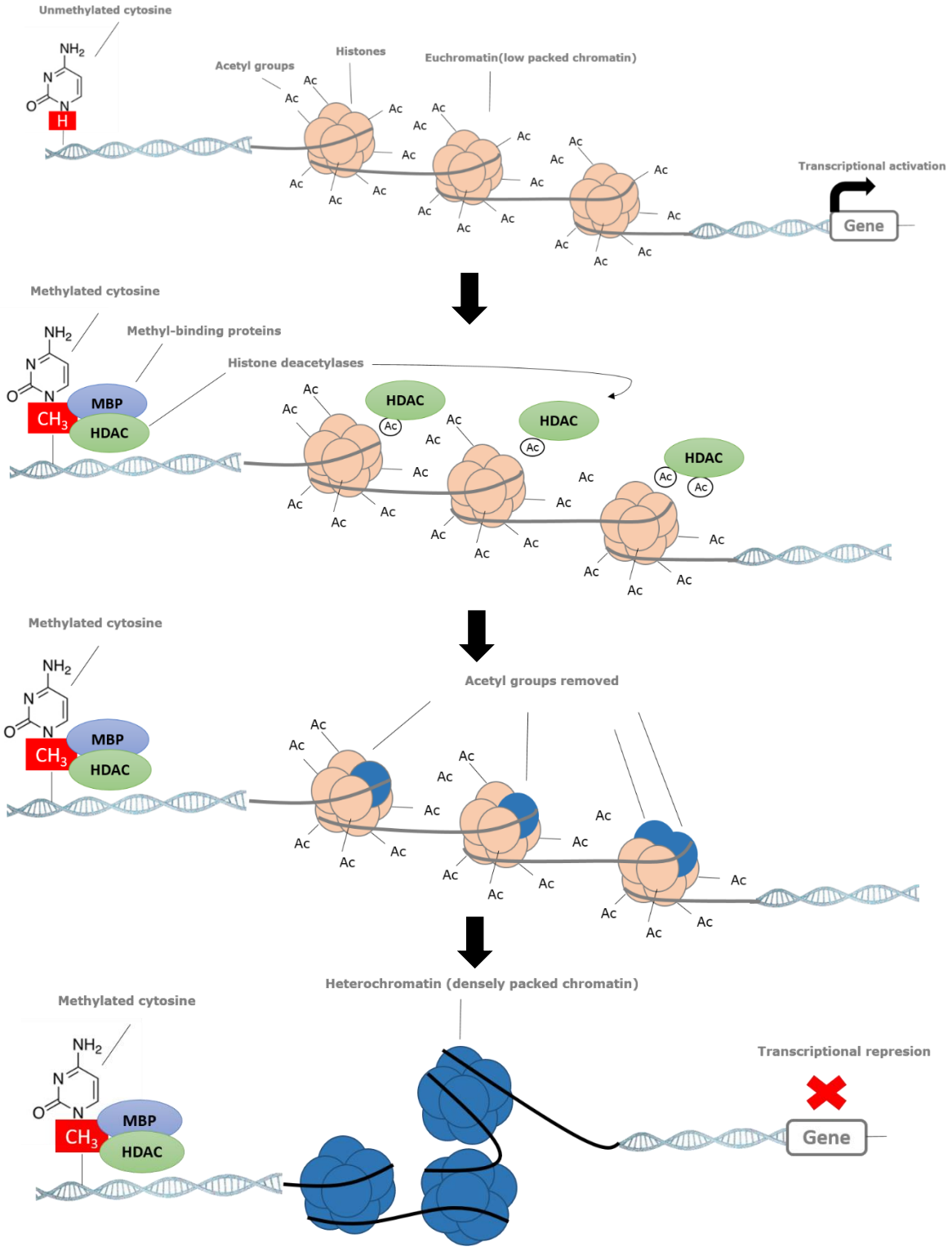


Figure 9: Scheme of how methylation reduces gene expression. Unmethylated cytosine and acetylated histones are associated with transcriptional activity. Acetylation of histones allows chromatin to be loosely packed. Once cytosine becomes methylated, methyl-the acetyl groups which induces the formation of densely packed chromatin (heterochromatin). This physically blocks transcription factors binding to receptors and is most commonly associated with repressed gene transcription

Although the role of DNA methylation has been reported in other pathological states, so far, only a few studies have demonstrated the potential role of DNA methylation and the activity and expressional levels of DNMTs in pain. The level of DNA methylation is controlled by both DNMTs and demethylation enzymes (e.g., ten-eleven translocation dioxygenases). Nerve injury induces increased DNMT3a mRNA and protein expression in injured DRG neurons and represses the expression of Orpm1 and Oprk1 genes which encode for MOR and KOR (Shao *et al*., 2017; Sun *et al*., 2017). Blocking this increase with a DNMT3a shRNA adenoviral vector, restores MOR and KOR expression and the morphine analgesic effects. Besides, increased DNMT3a expression in the injured DRG neurons by spared nerve ligation (SNL) promotes the decrease of the voltage-dependent potassium channel subunit Kcna2 (Zhao *et al*., 2017). Given that Kcna2 is a key player in neuropathic pain genesis, DNMT3a likely acts as an endogenous contributor to neuropathic pain development in injured DRG. Recent studies revealed that nerve-injury induced decreases in mu opioid receptor MOR, kappa opioid receptor KOR and Kv1.2 may be attributed to an increase in the level of the DNMT3a protein in the ipsilateral DRG neurons (Zhou *et al*.,2014; Sun *et al*.,2017; Zhao *et al*.,2017). Therefore, it is essential to understand the molecular mechanism of how DNMTs are regulated in the DRG following peripheral nerve injury. On the other hand, the expression of the isoform DNMT3b is also upregulated fourfold after nerve injury in adult DRG rats (Pollema-Mays *et al*., 2014).

Methyl-CpG-binding domain (MBS) proteins such as MeCP2, which are repressor proteins that bind directly to one or more methylated CpGs of a gene promotor are also involved in chronic pain. These proteins recruit other transcriptional co-repressors such as HDACs to the promoter region of the targeted gene for its silencing (Turek-Plewa and Jagodzinski, 2005). The expression levels of MeCP2 and the levels of its phosphorylation are increased in the SDH under chronic inflammatory pain conditions (Tochiki *et al*.,2012) and an increase in MeCP2 expression was seen in the SDH following chronic constriction injury (CCI) of sciatic nerve (Wang *et al*.,2016). In addition, system MeCP2 overexpression reduced both acute and neuropathic pain (Zhang *et al*., 2015). A more recent study has proved that expression of the MBS protein MBD1 in the DRG is required for the genesis of acute and

neuropathic pain probably by regulating DNMT3a-controled Oprm1 and Kcna2 gene expression (Mo *et al*., 2018). These authors proved that MBD1 is required for the development and maintenance of neuropathic pain in a mouse model of SNL-induced neuropathic pain. Overexpression of MBD1 protein lead to pain hypersensitivity though a negative regulation of Oprm1 and Kcna2 genes in the SDH and DRG of mice exposed to neuropathic pain.

HATs affect chemokine expression whereas HDACs affect cytokine expression within glial and macrophage cells that are reactive to neuronal damage. However, there is no agreement regarding histone and DNA methylation effects on inflammatory mechanisms that sustain pain states. Although increasing interest is shown within this epigenetic field, we are still at the initial steps of understanding these processes. Thus, further research needs to be performed to evaluate novel therapies that might be effective on patients that suffer from neuropathic conditions.

2.3 Histone modifications

As it was described in **chapter 2.1**, histones are small proteins with a positive charge that associate with the DNA (which is negatively charged) in the nucleus, helping to condense it into chromatin. The main types of histones involving in compacting DNA are histone H1, H2A, H2B, H3 and H4. Histones undergo post-translational modifications that alter their interaction with the DNA and nuclear proteins. Histones have long protruding N-terminal tails which can be covalently modified. There are various types of modifications of the tails including acetylation, methylation, phosphorylation, ubiquitination and sumoylation. The two main histone modifications are histone acetylation and methylation which are the consequences of the addition of acetyl and methyl groups mainly to arginine (R) or lysine (K) residues of histone tails.

2.3.1 Histone methylation

Histone methylation is mediated by histone methyltransferases (HMTs), including lysine methyltransferases (KMTs) and arginine methyltransferases (PRMTs) and histone demethylation by histone demethylases (HDMs). Methylation of histones takes place mainly in the lysine or arginine residues located in the histone tails (Greer and Shi, 2012). Histone methylation influences the recruitment and binding of different regularity proteins to

chromatin (Hyun *et al*., 2017; Kanistan *et al*., 2018). HMTs can transfer up to three methyl groups from the cofactor SAM to lysine or arginine residues of the histones (Morera *et al*., 2016; Kanistan *et al*., 2018). KMTs are more specific than histone acetyl transferases (HAT) and they generally target a specific lysine residue. The most frequent methylation epigenetic marks in histones of heterochromatin are H3K9, H3K27 and H4K20, all of them associated with gen silencing and chromatin packaging (inactive) (Torres and Fujimori, 2015). On the other hand, trymethylation of H3K4 and H3K36, as well as methylation of arginines in positions H4R3 and H3R2 associate with transcriptionally active promoters (Wysocka, 2006; Kouzarides, 2007; Zippo *et al*., 2009). Methylation of H3K4 residue is mediated in mammals by KMTs such as KMT2A/MLL1 and KMT2/MLL2. On the other hand, KMTs responsible for H3K9 methylation include Suv39h1, Suv39h2 and G9a. H3K36 methylation is catalyzed by KMT3B/NSD1, KMT3C/SMYD2 or KMT3A/SET (D) 3 (Morera *et al*., 2016; Hyun *et al*., 2017; Kaniskan *et al*., 2018).

2.3.2 Histone acetylation

Another important factor in the configuration of the heterochromatin is histone acetylation. Histone acetylation status is regulated by two groups of enzymes exerting opposite effects, histone acetyltransferases (HATs) and histone deacetylatses (HDACs). HAT catalyze the transfer of an acetyl group from acetyl-CoA to an amino acid group of the target lysine residues in the histone tails, which leads to the removal of a positive charge on the histones, weakening the interaction between histones and DNA (with negatively charged phosphate groups). This in turn typically makes the chromatin less compact and thus more accessible to the transcriptional machinery. HDACs remove acetyl groups from histone tail lysine residues, which results in packaging of chromatin and prevents the contact of transcription factors to DNA, thereby working as repressors of gene expression (Fierz *et al*., 2012; Swygert *et al., 2014;* Harb *et al*., 2015; Ceccacci *et al*., 2016). HATs are classified into five families; GCN5-related N-acetyltransferase (GNAT), MYST, KAT3A, KAT3B.

2.3.3 Histone modifications and disease

Histone methylation dynamics is known to have important role in many biological processes, including cell cycle regulation, DNA damage and stress response, development and differentiation (Yang *et al*., 2018; Millán-Zambrano *et al*., 2018). Aberrant expression of histone modifications has been associates to some of the most frequent human pathologies. Mutations in or altered expression of histone methyl modifiers and methyl-binding proteins correlate with increased incidence of various different cancers. For example, H3K27me3 methyltransferase EZH2 is upregulated in a number of cancer including gastric cancer (Zhao *et al*., 2018), renal cell carcinoma (Sun *et al*., 2018), prostate cancer (Zheng *et al*., 2018), colorectal cancer (Huang *et al*., 2018) and cervical cancer (Yang *et al*., 2018). Moreover, cell from patients with rheumatoid arthritis show an increase in H3K4me3 levels and a reduction on the levels of H3K27me3 (Messemaker *et al*., 2017). Furthermore, certain genes believed to be involved in systemic lupus erythematosus display altered H3K4me3 levels (Zhang *et al*., 2018).

2.3.4 Histone modifications and pain

Histone acetylation in the spinal cord has recently been implicated in nociceptive sensitization in animal models of neuropathic pain. Several studies suggest that modifications in histone H3 and H4 tails produce the aberrant transcription of cytokines and chemokines, being the reason of chronic inflammatory diseases (Hahn *et al*., 2008; Villagra *et al*., 2010).

The induced expression of chemokines and their receptor in infiltrated macrophages and neutrophils on the lesioned nerve has been shown to be related with an increased H3K9Ac and tri-methylation of H3K4 and their promoters (Kiguchi *et al*., 2013, 2014). Furthermore, another study observed an increased expression of CXCR1 and CCL1 chemokines by acetylation of H3K9 in the spinal cord, being responsible of neuropathic pain induced after injury (Sun *et al*., 2013). Similarly, recent studies have shown that HDAC inhibitors can alleviate inflammatory pain (Chiechio *et al*.,2009; Bai *et al*.,2010; Zhang *et al*.,2011) and attenuate the development of hypersensitivity in models of neuropathic pain (Zhang *et al*.,2011; Denk et al.,2013; Capasso *et al*.,2015). It has been described that neuropathic pain

maintenance involves HDAC1 since the use of specific HDAC1 inhibitor (LG325) ameliorated mechanical allodynia of SNI mice. Nerve injury increases HDAC as well as hypoacetylation of H3K9 within microglia of the SDH (Kami *et al*., 2016). In another study, administration of Baicalin, a natural compound, reversed H3 and HDAC1 expression in spinal cord, paralleled by a decrease of neuropathic pain after SNL (Cherng *et al*., 2014). Conversely, the HDAC1-HDAC6 inhibitor LG322, showed a less favorable antinociceptive profile (Sanna *et al*., 2017). Since HDACs inhibitors have demonstrated suppression of cytokine expression (Leoni *et al*., 2005; Kukkar *et al*., 2014;Khangura *et al*., 2017), decreased neuropathic pain through HDAC inhibitors may be related to suppression of inflammation through pro-inflammatory cytokine suppression.

Although histone methylation is an important epigenetic modification whose deregulation is involved in some pathological conditions, the role of histone methylation in chronic pain is still unclear. Histone methylation could repress or activate gene transcription depending on the sites that are methylated. In general, methylation of histone H3 at Lys9 or Lys27 (H3K9 or H3K27) or histone H4 at Lys20 (H4K20) correlates with transcriptional repression, whereas methylation of H3K4, H3K36 and H3K79 correlates with enhanced transcription (Kouzarides, 2007). Nerve injury produces the upregulation of G9a, an H3K9 methyltransferase responsible of gene silencing and it has been clearly demonstrating that contributes to transcriptional repression in primary sensory neurons, contributing to neuropathic pain (Liang *et al*., 2016). Other studies demonstrated that targeting G9a reverses the silencing of the Orpm1 gene, which encodes the mu opioid receptor (MOR) gene, and restores the effect of morphine on the hypersensitivity induced by peripheral nerve lesions (Zhou *et al*., 2014; Zhang *et al*., 2016). Nerve injury also increases the enrichment of H3K9me2 in the promoters of potassium channels, producing their silencing (Laumet *et al*., 2015).

Peripheral nerve injury induces a reduction in the H3K27me3 in the gene promoter of some cytokine genes related to neuropathic pain (Kiguchi *et al*., 2013). An increase in global histone methylation was also observed in the spinal cord after intrathecal injection of pertussis toxin, which induced significant thermal hyperalgesia (Tsai *et al*., 2012). After traumatic injury,

there is altered expression of the histone methyltransferase EZH2, the histone demethylase JMJD3 and the transcriptional repressor MeCP2. EZH2 catalyzes de di- and tri-methylation of histone H3 on lysine 27 (H3K27me2/3), which is a repressive biomarker that induces chromatin compaction and gene silencing. The expression levels of this KMT are increased in the neurons of the SDH in SNL rats with neuropathic pain (Yadav and Weng, 2017). Inhibition of EZH2 attenuates the expression of inflammatory mediators and the development and maintenance of mechanical and thermal hyperalgesia in rats with partial sciatic ligation (PSL). Genes found to be decreased by EZH2 were linked to cytokines, chemokines, enzymes and transcription factors (Arifuzzaman *et al*., 2017). The histone demethylase that specifically demethylates H3K27me2/3 producing de-repression, JMJD3, seems also to be involved in inflammatory mechanisms which contribute to the physiopathology after CNS injury. For example, it has been described that SCI produces an increase of JMJD3 expression in endothelial cells, inducing an increased expression of the cytokine IL-6 by demethylating its promoter (Lee *et al*., 2012). Although more evidence points to an important role for histone methylation in neuropathic pain, whether nerve injury-induced changes in histone methylation contribute to neuropathic pain remains to be investigated.

2.4 Non-coding RNAs: microRNAs (miRNAs)

The term non-coding RNA (ncRNA) refers to the functional RNA molecules that are transcribed from DNA but not translated into proteins. Abundant and functionally important types of ncRNA include transfer RNAs (tRNA) and ribosomal RNAs (rRNAs), as well as small RNAs such as microRNAs (miRNAs), small nucleolar RNAs (snoRNAs) and short interference RNAs (siRNAs). Recently, some ncRNA have been included as an epigenetic mechanism as they control gene expression. Epigenetic related ncRNAs include miRNAs, siRNAs, piRNAs and lncRNAs.

2.4.1 Characteristics and biogenesis of miRNAs

MicroRNAs (miRNAs) are a type of ncRNAs were discovered in 1993 during the characterization of the genes that control the temporal larval development in the nematode *Caernohabditis elegans (C.elegans)*. Two small

regulatory RNAs, *lin*-4 and *let*-7, were found. These genes did not encode proteins, but instead small RNAs with a key role in development (Lee *et al*., 1993; Reinhart *et al*., 2000). Later, it was proved that lin-4 and let-7 represent a widely extended type of small endogenous RNAs present in nematodes, flies and mammals and were subsequently called microRNAs (miRNAs) (Lee and Ambros, 2001; Lagos-Quintana *et al*., 2001; Lau *et al*., 2001). Since its discovery, hundreds of miRNAs have been identified well conserved in animals, plants, virus and many other species (Krol *et al*., 2010; Kozomara and Griffiths-Jones, 2011).

miRNAs are small single-stranded non coding RNAs of approximately 19-21 nucleotides in length that regulate gene expression post-transcriptionally. Usually, this regulation is carried out in two different mechanisms: target messenger RNA (mRNA) degradation or translational repression of the target mRNA (Fabian *et al*., 2009). The final result is a reduction in the target protein expression (Hobert, 2008). miRNAs are thought to regulate approximately 30% of the human genome, controlling the expression of genes involved in several biologic processes, including apoptosis, survival, cellular proliferation, differentiation and metastasis (Ambros, 2004; Bartel, 2004; Kloosterman and Plasterk, 2006; Valencia-Sánchez *et al*., 2006; Gangaraju and Lin, 2009; Li and Jin, 2010; Filipowicz *et al*.,2015). Nowadays, more than 4076 miRNAs have been identified in the human genome according to the last version (v7.0) of miRBase, the central resource for miRNA curation (Chou *et al*.,2018) and changes in the expression pattern have been associated with human pathologies (Piedade *et al*.,2016; Leggio *et al*.,2017).

Approximately, 40% of the identified human miRNAs are intragenic intronic (located within their host genes that codify for proteins) and 42% are intergenic (located between the genes that codify for proteins) (Rodriguez *et al*., 2004; Zeng, 2006; Wang, 2010). In the first case, expression of miRNAs can be correlated to the transcriptional regulation of the host gene, explaining thus the tissue specificity due to the expression of different groups of genes (Lin *et al*., 2006; Bartel, 2009). In the second case, the expression of miRNAs is regulated independently by its own regulatory elements (Zeng, 2006). MiRNAs could also come from repetitive sequences of DNA (Smalheiser and Torvik, 2005).

Mature miRNAs come from a series of steps that took place firstly in the nucleus and end in the cytoplasm, where they carry out their function. Most of the miRNA are transcribed by RNA polymerase II (Pol II) that produces a stemloop structure containing a big molecule called primary miRNA (pri-miRNA) which can range in size from hundreds of nucleotides to tens of kilobases (Lee *et al*.,2004). This molecule is cleaved in the nucleus by the RNAse III known as Drosha and its partner DGCR8 (DiGeorge syndrome critical region gene 8) in mammals or Phasa (in Drosophila and C.*elegans*) forming a multiprotein complex called the Microprocessor (Lee *et al*., 2003; Denli *et al*., 2004). This complex cleaves the pri-miRNA, producing an asymmetric cut that leads to a molecule of about 60-70 nucleotides called miRNA precursor (pre-miRNA). This molecule, that has two overhanging nucleotides in the 3' end is recognized by Exportin 5 and subsequently exported to the cytoplasm via Ran-GTP-dependent mechanism (Yi *et al*.,2003; Lund *et al*.,2004) (**Figure 10**).

Figure 10: Structure of the human pri-miRNA-30 RNA hairpin. Drosha cleavage sites are shown by arrows and DICER cleavage sites by triangles (Taken from Cullen, 2004).

Once in the cytoplasm the pre-miRNA is processed by a cytoplasmic endonuclease RNAse III called DICER, leading to a double-stranded miRNA structure known as miRNA duplex (Bohnsack *et al*., 2004; Lund *et al*., 2004). One of the chains of this miRNA duplex, known as miRNA* is cleavaged by fragmentation or a bypass mechanism (Khvorova *et al*., 2003). The other chain, the mature miRNA, that has a longitude of about 20-25 nucleotides is incorporated in the ribonucleoproteic complex miRISC (miRNA-induced silencing complex), which constitutes the catalytic machinery responsible for the degradation of the target mRNA and/or the inhibition of the translation (Zeng, 2006; Bartel, 2009; Krol *et al*., 2010). Known elements of the human miRISC complex include DICER, Argonaute (AGO) proteins, TRBP (HIV-1 transactivation responsive element TAR RNA-binding protein) and PACT (a double-stranded RNA-binding protein). AGO is the catalytic enzyme that cuts the target mRNA and it is necessary for the production of the mature miRNA,

having a key role in the miRNAs biogenesis. In mammals, four homologous have been identified (AGO1-AGO4), being AGO2 the only one with endonucleotic activity, cuting the target mRNA (Liu *et al*., 2004; Meister *et al*., 2004). AGO proteins contain two RNA-binding domains: a PAZ domain that binds to the 3' monocatenary ending of the mature miRNAs (Song *et al*., 2003; Yan *et al*., 2003; Lingel *et al*.,2004) and a PIWI domain that binds to the target mRNA (Ma *et al*., 2005; Parker *et al*., 2005; Mallory and Bouche, 2008). MiRNAs can negatively regulate the genetic expression by two main mechanisms: the degradation of the target mRNA or the repression of the translation (Zeng, 2006; Bartel, 2009). Once the miRNA has been incorporated to the miRISC complex, the mature miRNA guides the complex to the target mRNa by base pairing. The use of one or the other mechanism will depend on the degree of complementarity between the miRNA and its target mRNA. If the complementarity is high or total, the mRNA will be cut and degraded whereas if the complementarity is not total or not enough, translation of the mRNA will be inhibited (He *et al*., 2004). After the degradation of the target mRNA, the miRNA remains intact and can guide the miRISC complex towards the recognition and degradation of other mRNAs (**Figure 11**).

According to this, a miRNA can act on many 3'UTR regions of a wild number of genes. Studies in microarrays prove that miRNAs can inhibit many target mRNAs that contain complementary sequences to the 2-7 positions of the 8' ending of a miRNA, region known as seed sequence (Brennecke *et al*., 2005; Lewis *et al*., 2005; Chen and Rajewsky, 2006; Grimson *et al*., 2007).

2.4.2 miRNAs and disease

As discovery of human miRNAs increased, the research focus was gradually shifted towards the functional characterization of miRNAs, particularly in the context of human diseases. miRNA expression patterns are tissue-specific and in many cases define the physiological nature of the cell (Lagos-Quintata *et al*., 2002; Lim *et al*., 2005). Many genetic studies have proved that expression of miRNAs is altered in a wild number of human diseases including cancer, metabolic diseases, viral infection, neurodegenerative disorders…etc. More than 70 human diseases have been associated with altered levels of miRNAs (Pang *et al*., 2010; Sayed and Abdellatif, 2011; Liu and Xu, 2011;

Watahiki *et al*., 2011; Anderson *et al*.,2015; Qabaja *et al*., 2013; Zhang and Banerjee.,2015; Feng *et al*.,2018). They are being considered for both clinic diagnostic and as a possible therapeutic target (Hammon, 2015).

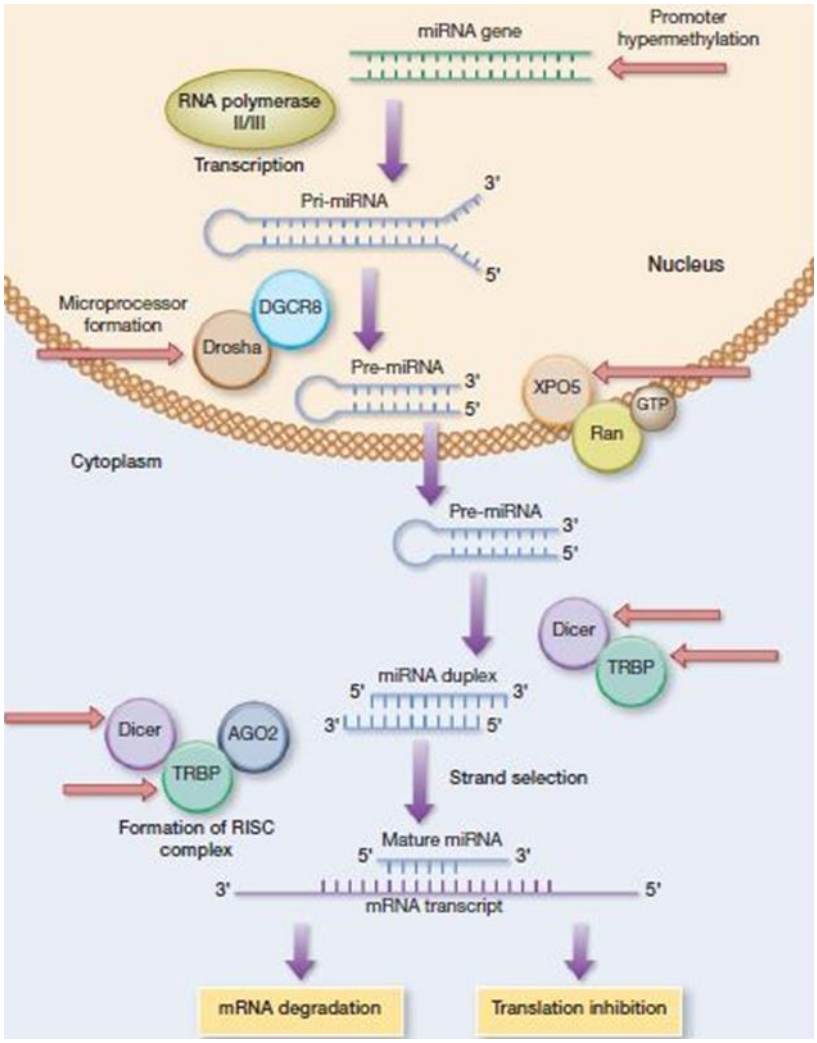


Figure 11: miRNA biogenesis pathway. miRNA genes are transcribed by RNA Pol II or Pol III to generate pri-miRNas whose hairpin structures are cleaved by Drosha/DGCR8 to release pre-miRNAs. Pre-miRNAs are exported from the nucleus by Exportin 5-RanGTP into the cytoplasm. Once in the cytoplasm, pre-miRNAs are further processed by DICER/TRBP to form a 22 nucleotides duplex. One strand is then selected to function as a mature miRNA, and the other strand is degraded. (Taken from Mulrane *et al*., 2013).

miRNAs are known to play a crucial role in vital biological processes such as embryonic development, maintenance of pluripotent stem cells in the embryo, tissue and neuronal development, cell dead and division, cell metabolism, intracellular signal, immunity and cell migration…etc (Stappert *et al*.,2015; Afzal *et al*.,2016; Kim *et al*.,2016; Naaman *et al*.,2017).

Cancer has been the most prominent of human diseases with a clear role for miRNA regulation. Calin *et al* demonstrated in 2005 a frequent deletion of miR-15 and miR-16 among 65% of the B-cell chronic lymphocytic leukemia patients. Since that moment, it has been established correlations between aberrant miRNA expression patterns and increased occurrence of different cancers; brain, gastric, lung, colon, pancreas, testis, breast, renal, thyroid and ovarian (Syring *et al*., 2015;Kim *et al*.,2016; Hu *et al*.,2017;Özata *et al*.,2017; Yang *et al*.,2017;Yu *et al*.,2018; Zhou *et al*.,2018). miRNAs also play key roles in tumor invasion and metastasis (Paladini *et al*.,2016; Guo *et al*.,2017; Chen *et al*.,2018). These cancer-related miRNAs are potentially useful for developing not only early diagnosis but also novel anti-cancer strategies.

miRNAs have been discovered in multiple virus species as well. Viral-encoded miRNAs have been identified in the Epstein-Barr virus, papilloma virus, adenovirus, hepatitis C virus, herpes virus, human immunodeficiency virus (HIV) and Ebola virus (Majer *et al*.,2017; Skinner *et al*.,2017; Yu *et al*.,2017).

Altered levels of miRNAs have been associated also with many common autoimmune-related diseases, including multiple sclerosis, systemic lupus erythematosus, type I and II diabetes, psoriasis, asthma, nonalcoholic fatty liver disease and Crohn's disease (Andersen *et al*., 2015; Cerdá-Olmedo *et al*., 2015; Gong *et al*., 2015; Svitich *et al*., 2018; Watkin *et al*., 2018).

Studies with microarrays have identified a large number of miRNAs that are expressed in the heart. For example, miR-1 and miR-133 are highly enriched in the heart, skeletal muscle and thus have a crucial role in cardiac development, regulation of key factors for cardiogenesis and the hypertrophic development response (Lagos-Quintana *et al*., 2002). Many studies have pointed the evidence that miRNAs are involved in cardiac pathologies such as; failing human heart, ischemic heart disease, cardiac hypertrophy, childhood dilated cardiomyopathy were miRNAs aberrant expression has been shown (Corsten *et al*., 2010; Fichtescherer *et al*., 2010; Matkovich *et al*., 2010; Zampetaki *et al*., 2010; Kuwabara *et al*., 2011; Jiao *et al*., 2017). Studies in our lab support the emerging role for miR-21 and miR-133a as cardiac fibrosis regulator and its putative value as biomarkers with

therapeutic potential (Villar *et al*., 2013; García *et al*., 2013; García *et al*., 2015).

miRNAs have been linked to several metabolic pathways including cholesterol and fatty acid metabolism and transport, hypertension, pancreatic islet function and glucose metabolism, adipogenesis and adipocyte differentiation (Frost and Olson, 2011; Nandukumar *et al*.,2017; Zaiou *et al*.,2018; Kang *et al*.,2018). This suggests that miRNA-based therapies could lead to new treatments of obesity and diabetes.

Recent studies have demonstrated that miRNAs are not exclusively intracellular, but also extracellular. miRNAs are present in a cell-free circulating form in many different biological fluids, including saliva, serum and plasma where they can be easily detected and measured (Fehlmann *et al*.,2016). This characteristic makes miRNAs very attractive molecules from a clinic point of view. 90% of the miRNAS are associated with proteins (vesicle-free) such as AGO2 and 10% are held inside small membranous vesicles such as shedding vesicles or exosomes (Hébert *et al*., 2009; Jin *et al*., 2013). Importantly, extracellular circulating miRNAs have been found aberrantly expressed in the bloodstream during the course of many diseases (Turchinovich *et al*.,2016), and some evidences suggested a potential role for miRNAs in cell-cell communication during pathological processes, particularly those packaged onto exosomes (Valadi *et al*.,2007; Momen-Heravi *et al*.,2015). Despite intense research, the origin of circulating RNAs remains poorly understood. Circulating miRNAs are extremely stable and can be detected with high specificity and sensibility in different body fluids such as serum, plasma, saliva, orine, mother milk and tears. Recently, numerous studies have shown that circulating (plasma/serum) levels of many miRNAs were significantly altered in different diseases (Chugh *et al*.,2013; Vignier *et al*.,2013; Trebicka *et al*., 2013; Foye *et al*.,2017; Tigchelaar *et al*.,2017). For example, seric levels of miR-141 have been used as diagnostic marker to differentiated prostate cancer patients from healthy individuals (Mitchell *et al*., 2008); relation between miR-126 and miR-182 in urine samples can be used to detect veggie cancer (Hanke *et al*., 2010) and a decrease in levels of miR-125a and miR-200a in saliva samples is associated with oral carcinoma of squamous cells (Park *et al*., 2009). Extracellular circulating miRNAs offer

some potential advantages as informative biomarkers compared to blood biomarkers based on proteins, as most of the circulating miRNAs can be easily detected by quantitative PCR. For example, miR-21 has been recently discovered as a potential biomarker of cardiac injury in rats (Gryshkova *et al*., 2018), high levels of miR-200c have been associated with good survival rates in endometrial cancer (Wilcznski *et al*., 2018). The high expression specific levels of some miRNAs such as miR-122 in the liver (Trebicka *et al*., 2013) and miR-499 in the heart (Adachi *et al*., 2010), will imply the possibility to use seric levels of these miRNAs to control the "health" of specific organs.

With the technique of the quantitative polymerase chain reaction (qPCR), important levels of miRNAs in the cerebrospinal fluid (CSF) have been identified in patients with primary lymphoma of CNS, as for example miR-21, miR-19 y miR-92a. Those miRNAs are also involved in brain inflammatory processes and other neurological diseases (Baraniskin *et al*., 2011). These characteristics have focus the attention to reveal its potential use as bed biomarkers with diagnostic and prognostic value in numerous pathologies (Bali *et al*., 2014). Recent results in our lab suggest the relation between the circulating expression levels of miR-21 and miR-133a, and myocardic fibrosis in patients (García *et al*., 2013, Villar *et al*., 2011 and 2013) as well as a correlation between miR-30c-5p increased expression in plasma and CSF in patients with neuropathic pain associated with leg ischemia compared to control patients without pain (Tramullas *et al*., 2018).

2.4.2.1 miRNAs in the nervous system

miRNAs are abundant and are expressed in a specially and temporally manner in the CNS, where they are involved in numerous neurobiological processes including neurogenesis, neural differentiation, synaptic plasticity and cell responses that occur in the pathologic neural plasticity processes (Goldie and Cairns, 2012; Adlakha *et al*.,2014; Nieto-Díaz *et al*.,2014; Elramah *et al*., 2014; Jin *et al*.,2016). Neural miRNAS are involved in some steps during synaptic development including dendritogenesis, synaptogenesis and synaptis maturation (Agostini *et al*., 2011; Adlakha *et al*., 2014; Störchel *et al*., 2015). They are also involved in the glia differentiation. For example, miR-24 is one of the well-known miRNAs that are involved in neuronal

differentiation. It has been shown to be expressed in neurons, but not in astrocytes and its levels increase over time in the developing nervous system. Recently, miRNAs have also been shown to be critical regulators of oligodendrocyte differentiation and myelination in the vertebrate CNS (Kuypers *et al*., 2016; Schafferer *et al*., 2016). For example, overexpression of miR-219 and miR-338 in the spinal cord is enough to promote oligodendrocyte differentiation.

In human, disease-specific miRNAs profiles have been detected in the CSF in many neurologic diseases and could be used as biomarkers in Parkinson disease, Alzheimer disease, Huntington disease, or epilepsy (Burgos *et al*., 2014; Gui *et al*., 2015; Reed *et al*., 2018).

Recent human studies show that some miRNAs play a crucial role in genetic expression alterations associated to inflammatory pathologies (Alevizos and Illei 2010; Yu *et al*., 2011; Gheinani *et al*., 2013), for example, miR-146 has an effect on chronic pain in osteoarthritis, as it regulates inflammatory response of Toll-like receptors and nuclear factor kappa B (Li *et al*., 2011). Furthermore, in patients with regional complex pain the correlation between miRNAs patterns in blood and inflammatory markers has been a useful tool to establish criteria for stratification of the patients (Orlova *et al*., 2011; Li *et al*., 2011), the same occurs in patients with fibromyalgia (Cerdá-Olmedo *et al*., 2015).

2.4.2.2 miRNAs and neuropathic pain

Several miRNAs have been observed to change their expression in models of neuropathic pain. The results obtained indicate that many miRNAs undergo expression changes in individual pain conditions and recent studies in animal models show that alterations in the expression of specific miRNAs in the SNC contribute to the pathologic plasticity that underlies pain.

For instance, miR-7a is the most robustly decreased miRNA in the injured DRG, and is associated to neuropathic pain through regulation of neuronal excitability. Overexpression of this miRNA suppresses established neuropathic pain and its downregulation is enough to cause pain-related behaviors in healthy rats. miR-7a targets b2 subunit of the voltage-gated sodium channel (Sakai *et al*.,2013). Sodium voltage gated channels have

been observed to be regulated by other miRNAs. For example, miR-96 inhibits Nav1.3 expression and alleviates neuropathic pain after CCI (Chen *et al*., 2014). Similarly, miR-30b controls the expression of Nav1.7 after SNI. miR-30b over-expression in spared nerve injury rats inhibits SCN9A transcription, resulting in pain relief. In addition, miR-30b knockdown significantly increased hypersensitivity to pain in naïve rats (Shao *et al*., 2016). It has been established also, that the cluster of microRNAs that includes miR-96, miR-182, and miR-183, has a reduced expression in primary afferent DRG neurons in a model of SNL. The redistribution of microRNAs is associated with altered distribution of the stress granule protein TIA-1, which may have a significant impact on regulatory activity of microRNAs (Bhattacharyya *et al*., 2006; Vasudevan *et al*., 2007; Aldrich *et al*., 2009). Specifically, the microRNA-183 cluster in mice controls more than 80% of neuropathic pain–regulated genes and scales basal mechanical sensitivity and mechanical allodynia. For example, it controls voltage-gated calcium channel subunits α2δ-1 and α2δ-2 and TrkB+ light-touch mechanoreceptors (Peng e*t al*., 2017). Besides, another microRNA that controls calcium voltage-gated channels is miR-103, which regulates the expression of the three subunits forming Cav1.2-comprising L-type calcium channel (LTC). This regulation is bidirectional since knocking-down or over-expressing miR-103, respectively, up- or down-regulate the level of Cav1.2-LTC translation. Besides, miR-103 knockdown in intact rats results in hypersensitivity to pain. This miRNA, is downregulated in neuropathic pain animals, and its intrathecal administration relieve pain after SNL (Favereaux *et al*., 2011).

Another study in the chronic constriction injury model showed that miR-134 was up-regulated in the SDH at day 14 after surgery (Genda *et al*., 2013). Willemen *et al* also showed in 2012 that the level of miR-124 in microglia isolated from the spinal cord of inflamed rats was significantly lower than in wild-type animals. Furthermore, studies carried out in animal models of neuropathic pain by spinal cord contusion showed altered levels of many microRNAs in the spinal cord (Norcini *et al*., 2014; Strickland *et al*., 2014). Recent studies show the existence of alterations in the expression of some miRNAs (miR-96, miR-182 y miR-183, mir-30d, mir-30a, miR-125b, miR-379) in the DRG after spinal nerve ligation (Aldrich *et al*., 2009; Von Schack

et al., 2011; Bali *et al*., 2014). miR-23b has a crucial role in the neuropathic pain improvement by the inactivation of its target gene NOX4 and by the protection of the GABAergic neurons from cellular death (Im *et al*.,2012). In addition, miR-21 is overexpressed in neurons of the DRG in the late phase of neuropathic pain (Sakai *et al*., 2013)

Similarly, there are studies that prove the deregulation of several miRNAs using neuropathic pain models after the sciatic nerve partial ligation. A decrease in the expression levels of miR-1, miR-16 and miR-206 has been observed in the DRG without affecting the levels of expression in the SDH. In contrast, animals subjected to an axotomy or complete ligation of the sciatic nerved showed an increase in the expression levels of miR-1, miR-16 and miR-206, which was directly related to the time elapsed after the nerve injury. Animals subjected wo chronic constriction of the sciatic nerve showed a deregulation in the expression levels of miR-124, miR-494, miR-720, miR-690, miR-668, miR-500, miR-221 and miR21 in the spinal cord (Brandenburger *et al*.,2012; Genda *et al*.,2013) and an increase in the expression levels of miR-125b and miR-132 in the hippocampus (Arai *et al*.,2013; Hori *et al*.,2013).

The relation between miRNAs and neuropathic pain has not only been found in the animal models but also in patients. It has been reported an increase in the expression of miR-34c-5p, miR-107, miR-892b, miR-486-3p and miR-127-5p in patient serum with postherpetic neuralgia vs patients with acute herpes zoster (Huang *et al*., 2017).

The nociceptive behavior of the animals and their response to analgesic drug can be modulated by manipulating the expression levels of specific miRNAs with anti-miRs or synthetic miRNas (Niederberger *et al*., 2011; Nieto-Díaz *et al*., 2014). Therapeutic approaches have been developed aimed at normalizing the expression of miRNAs by using synthetic oligonucleotides (miR-mimics), in case of a reduced expression, or anti-miRNAs and antagomiRNAs in the case of an overexpression (Van Rooij *et al*., 2012; Kynast *et al*., 2013; Lötsch *et al*., 2013). All of this has led the biotechnology community to include miRNAs as preferred therapeutic targets (Kress *et al*., 2013; Tan *et al*., 2013; McDonald and Ajit, 2015). miRNA-targeted therapeutics showed some promising preclinical results (Willemen *et*

al.,2012; Yunta *et al*.,2012; Huo *et al*.,2013; Chen *et al*.,2014; Rupaimoole and Slack, 2017) and one miRNA entered clinical trial (Chakraborty *et al*.,2017).

2.4.2.3 microRNA-30c (miR-30c-5p)

The miR-30 family contains five members and six distinct mature miRNAs (miR-30a, miR-30b, miR-30c-1, miR-30c-2, miR-30d, miR-30e) and it is encoded by six genes located on human chromosomes 1,6 and 8. These miRNAs share the same seed sequence located near de 5' end, but have different compensatory sequences located near the 3' end. These differences allow miR-30 family members to target different genes and pathways and sometimes lead to totally opposite behaviors (Brennecke *et al*., 2005). The importance of this family was described originally in oncology, as the deregulation of some of its member is key for the production of some tumors (Zhao *et al*., 2014). The miR-30 family members that mainly function as tumor suppressors could cause tumor inhibition (Zhang *et al*., 2014), apoptosis (Yu *et al*., 2010) and cell cycle arrest through negative regulation of oncogenes and oncogenic pathways (Fu *et al*., 2014).

The function and deregulation of miR-30c has been related with multiple biological processes and pathologies including liver development (Hand *et al*.,2009), angiogenesis (Bridge *et al*.,2012), neuronal proliferation (Sun *et al*.,2016), cardiac hypertrophy (Raut *et al*.,2016), fibrosis (Yang *et al*.,2016), pulmonary hypertension (Xing *et al*.,2015), breast cancer (Tanic *et al*.,2012), prostate cancer (Huang *y cols.,* 2017),arterial thrombosis (Luo *et al*.,2016), obesity (Peng *et al*.,2014), cirrhosis (Wen *et al*.,2016) virus infection (Zhang *et al*.,2016)..etc.

miR-30c has also a protective effect in pain caused by medular ischemia in rats (Li *et al*.,2015). It is involved in hippocampal sclerosis (Nelson *et al*., 2015). A downregulation has been related with learning difficulties in mouse (Sun *et al*., 2016) and an upregulation in epilepsy (Alsharafi and Xiao, 2015). Recent work from our group shows the relation between miR-30c and neuropathic pain in rats subjected to sciatic nerve injury. The overexpression and silencing of miR-30c exhibit opposite effects over the development of allodynia after nerve injury. Administration of miR-30c mimic accelerates the

development of allodynia while antimiR-30c administration delays neuropathic pain development and reverses established allodynia after sciatic nerve injury in rats. Our group also provided evidence that the interaction between miR-30c-5p and its target TGF-β modulates the endogenous opioid system. In addition, the circulating levels of miR-30c in plasma and CSF and tissue in patients with critical leg ischemia suggest that measuring miR-30c-5p concentration in plasma and CSF might help to determine the likelihood to develop neuropathic pain (Tramullas *et al*., 2018).

2.5 Epigenetics mechanisms as therapeutic targets for chronic pain

Although available therapeutics are effective for acute pain relief, drugs used to control chronic pain are less efficacious, and the development of novel pharmacologic therapies have experienced little progress in recent decades. Chronic pain is predominantly treated with two classes of drugs: nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids. Both produce a number of adverse side effects and often fail to provide adequate long-term relief in many chronic conditions (Johnson and Greenwood-Van Meerveld, 2014). Therefore, the need for better therapeutic options for the management of chronic pain is evident. Epigenetics drugs offer the opportunity to modify the underlying pathology of chronic pain and not just attenuate the symptom. HDAC inhibitors and HAT inhibitor are the most commonly used epigenetic drugs in neuropathic pain studies. It has been demonstrated that partial sciatic nerve ligation-induced hypoesthesia could be prevented by administration of the HDAC inhibitors valproate and TSA (Matsushita *et al*., 2013). Another study showed that intrathecal administration of HDAC inhibitor MS-275 prevented SNL-induced mechanical and thermal hyperalgesia by increasing acetylation of H3K9 and altering HDAC1 expression in the SDG (Denk *et al*., 2013). Besides, HAT inhibitors such as anacardic acid after PSL decreased acetylation of H3K9 at the promoters of macrophage inflammatory protein 2 and CXC chemokine receptor type 2 in macrophages and neutrophils, thereby attenuating PSNL-induced thermal hyperalgesia (Kiguchi *et al*., 2013).Curcumin, which has known HAT inhibitory properties, has shown to effectively attenuate neuropathic pain by silencing pronociceptive genes Bdnf and cyclooxygenase (COX)-2 by reducing the

binding of p300/CREB-binding protein, H3K9ac and H4K5ac to their promoters (Zhe *et al*.,2014). The effects of targeting DNA methylation have also been issvala in the context of neuropathic pain models. For example, the use of 5-aza to antagonize DNMTs after injury attenuated mechanical and thermal hyperalgesia by reversing CCI-induced expression of methyl CpG binding protein 2 and global DNA methylation (Wang *et al*.,2011).

The concept of using epigenetic therapies for the treatment of chronic pain remains unproven but evidence to date suggests that this approach may offer more benefit to patients over the current analgesic regiments.

2.5.1 Analgesics as epigenetic modulator for chronic pain

Available analgesic drugs used to control chronic pain exert pain-relevant epigenetic effects and can be classified in different classes. They include cyclooxygenase (COX) inhibitors, opioids and other analgesic drugs.

Cyclooxigenase inhibitors

Long-term administration of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) including COX2 inhibitors celecoxib and sulindac might exert therapeutically relevant effects by epigenetic modulation. For example, aspirin inhibited DNA methylation of the FGF2 promoter and protected coronary artery endothelial cells from LDL-induced apoptosis (Chang *et al*., 2013). Calecoxib, which is an analgesic and anti-inflammatory drug for lon-term therapy of inflammatory pain in musculoskeletal diseases reversed tumour-induced hypermethylation of ESR1, which encodes the estrogen receptor in patients with colon cancer (Pereira *et al*., 2004; Shen *et al*., 2009).Besides, celecoxib increased histone H3 and H4 acetylation, resulting in reduced tumour growth in liver carcinoma (Cui *et al*.,2008)and also altered the expression of different miRNAs in human colorectal breast cancer (Saito *et al*.,2013; Wong *et al*.,2014).

Opioids

Opioids are the most frequently administered analgesics for severe pain and are associated with side effects on epigenetic mechanisms. For example, morphine reduces the expression oh histone-lysine-N-methyltransferase

EHMT2 (G9a) which leads to a decrease in the histone H3K9 dimethylation in the nucleus accumbens of mice, which might contribute to the development of morphine addiction (Sun *et al*., 2012). Also, patients with opioid addiction which received methadone substitution, methylation of global DNA and of OPRM1 (u-type opioid receptor) was increased in white blood cells. The increase observed in global methylation in patients treated with opioids might contribute to opioid-induced hyperalgesia (Doehring *et al*., 2013). Therefore, opioid are involved in diverse types of epigenetic regulation, and potentially contribute to analgesic effects but also to unwanted effects such as opioid-induced hyperalgesia or addiction.

Other analgesic drugs

Certain antidepressants (such as fluoxetine and amitriptyline) and some antiepileptic drugs have been used as a therapy for chronic neuropathic pain (Verdu *et al*.,2008; Dharmshaktu *et al*.,2012; Moore *et al*.,2014; Wiffen *et al*.,2014). Response to these groups of drugs associate with several epigenetic effects such as histone acetylation and DNA methylation in animal and cell models (Perisic *et al*.,2010; Mao *et al*.,2011; Wang *et al*.,2011; Schmidt *et al*.,2013).

Although the association between classic analgesics and epigenetic processes has not been reported in the context of nociceptive signalling pathways yet, the epigenetic effects of these drugs are likely to be direct rather than secondary effects or a consequence of pain relief.

3. Neuronal stress reaction after the injury of the axon

3.1 The cytoplasm

The cytoplasm that surrounds the nucleus of eukaryotic cells consists of the cytosol and the cytoplasmic organelles suspended in it. The composition of the cytoplasm depends on the cell phenotype. In the particular case of projection and sensitive neurons, the protein synthesis machinery is structurally organized forming parallel arrangements called Nissl bodies which are stacks of rough endoplasmic reticulum (RER) studded externally with ribosomes and interspersed with rosettes of free polyribosomes. Neurons require an important rate of synthesis of specific proteins to assure well-

working and survival, reason why they exhibit numerous and prominent Nissl bodies distributed through all the cytoplasm. Since most proteins are proteins modified for exporting or to be integrated into the intracellular system of endomembranes, Nissl bodies are very prominent and associate Golgi dictyosomes. The presence of numerous mitochondria organized among the Nissl bodies is justified since the translation and post-translational modifications are energy dependent (Pena *et al*., 2001).

Another essential component of neurons is the cytoskeleton, represented by the microtubules, organized in the perinuclear centrosomatic area. They are responsible for the rapid molecular traffic in neurites and intermediate filaments (neurofilaments) that guarantee somatic and nuclear morphology and polarization.

3.1.1 Neuronal Chromatolysis

Chromatolysis is a reactive response that occurs in the cell body of damaged neurons, involving the disruption, dispersal and redistribution of Nissl substance (rough endoplasmic reticulum and associations of free polyribosomes) leaving clear areas of empty cytoplasm (Bradley *et al*., 2018). Trauma (such as axonal transection or crushing), ischemia, toxicity or stress are the best-known causes of chromatolysis. Neural recovery though regeneration can occur after chromatolysis, but most often it is a precursor of apoptosis. Specifically, "central chromatolysis" is the most common form of chromatolysis and is characterized by a rounded neuronal cell body with loss or complete absence of Nissl substance from the center of the perikaryon towards the plasma membrane and an eccentric displacement of the nucleus towards the periphery of the cell (**Figure 12**). If the neuron survives and the demand for protein synthesis subsides, the cellular morphology returns toward normal by first passing through a stage of "peripheral chromatolysis" in which Nissl bodies reappear within the perikaryon in a central-to-peripheral pattern.

Central chromatolysis and nuclear eccentricity has been observed in the spinal anterior horn and motor neurons of neurodegenerative disorders such as amyotrophic lateral sclerosis (Martin, 1999; Riancho *et al*., 2014) and spinal muscular atrophy (Ito *et al*., 2011; Tapia *et al*., 2012; Tapia *et al*.,

2017). This functional change could result in the elimination of certain excitatory synaptic inputs and therefore give rise to the clinical motor function impairment that is characteristic of the ALS and SMA diseases. Chromatolysis has also been observed in neurons from Alzheimer's patients, often as a precursor to apoptosis (Joseph *et al*., 2007).

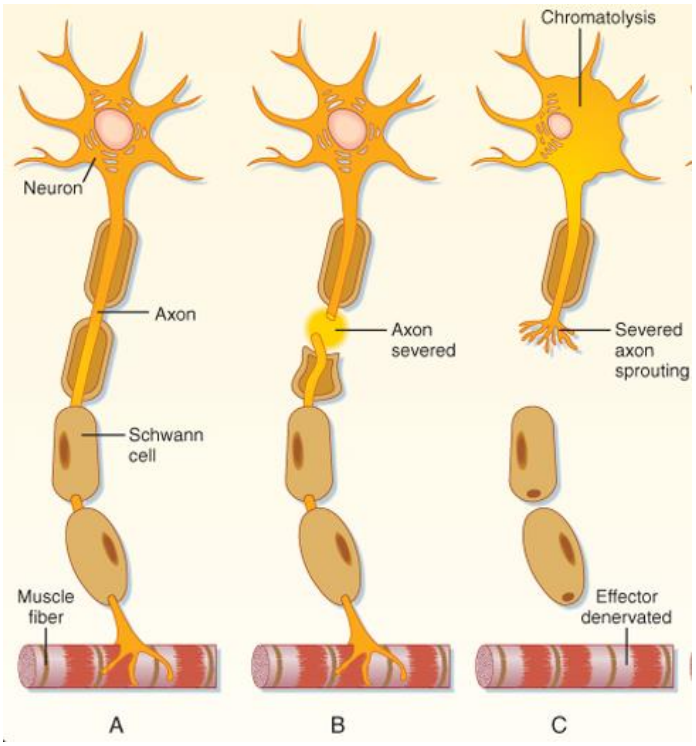


Figure 12. Scheme of a neuron undergoing chromatolysis. After a healthy neuron (**A**) suffers an injury to the axon (**B**), Nissl bodies suffer a disruption and migrate together with the nucleus towards the periphery of the cell leaving an empty cytoplasm (**C**). Because it cannot synthesize new proteins, the axon distal to the injury dies and all the associated synaptic endings disintegrate. Taken from Koeppen & Stanton: Berne and Levy Physiology, 6th Edition.

3.2 The nucleus

The nucleus is an organelle that is compartmentalized structurally and functionally in two fundamental domains: the chromosome territories, in which each chromosome occupies a specific nuclear subvolume, and the interchromosomal domain or inter-chromatin compartment (Cremer and Cremer, 2006; Cremer *et al*., 2006). In the interchromosomal domain are located the nucleolus and an important heterogeneous group of nuclear bodies lacking chromatin involved in the processing of mRNAs, the areas of splicing factors (nuclear speckles) and the nuclear Cajal bodies (Lafarga *et*

al.,2009; Moore and Proudfoot, 2009; Spector and Lamond, 2011) (**Figure 13**).

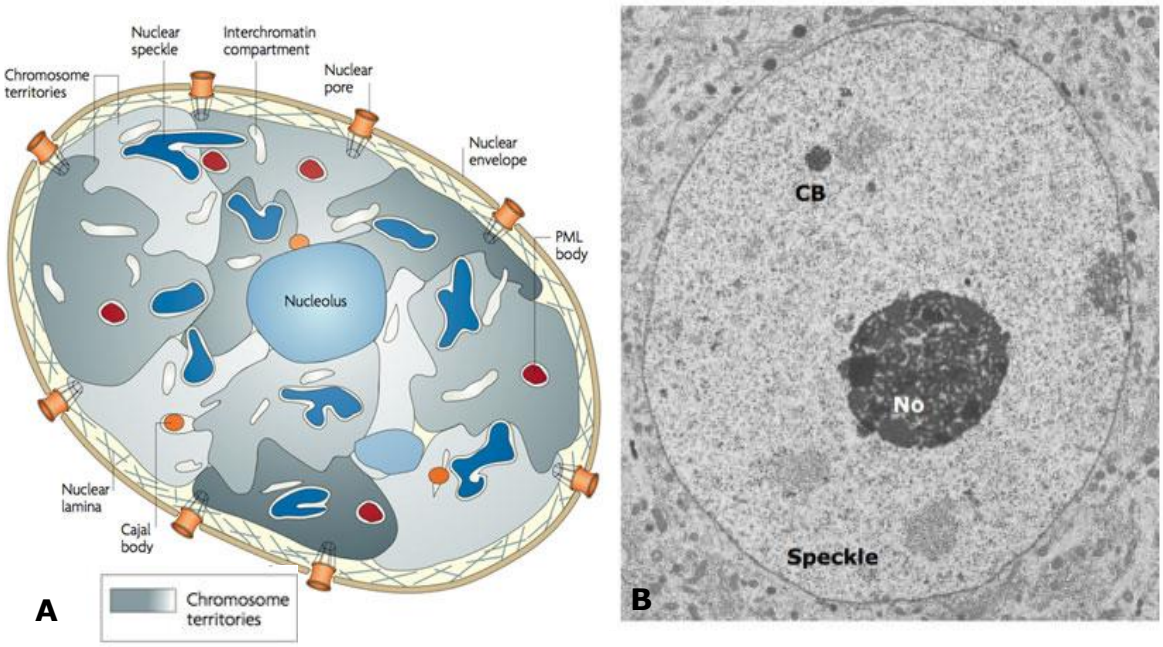


Figure 13: Nuclear compartments. A: The nucleus is characterized by a compartmentalized distribution of functional components. The nuclear envelope contains pores and the nuclear lamina. Chromatin is organized in distinct chromosome territories. Also depicted are nuclear speckles, promyelocytic leukemia bodies (PML) and Cajal bodies. **B**: Electron microscopy image showing a nucleus with some of the elements described in the previous scheme such as the nucleolus (No), the speckles and a Cajal body (CB). (Taken from Lanctot *et al*., 2007).

3.2.1 The nucleolus

The nucleolus is a non-membranous organelle whose functions are the organization of the "tandem" genes that constitute the ribosomal DNA (rDNA) and the biogenesis of the ribosomes. This process includes the synthesis and processing of rRNAs, as well as the assembly of the pre-ribosomal particles. These processes are intrinsically regulated to achieve the adaptation of proteostasis to the actual cellular translation demands. The level of activation and function of the nucleolus is reflected in the structural organization and distribution of its components: the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC) and the interstices (**Figure 14**). Each FC together with the DFC constitutes the functional unit where the biogenesis and initial processing of the rRNAs take place. The FC concentrates and stores components of the RNA polymerase I transcription

machinery, such as the upstream binding factor (UBF) and RNA polymerase I, involved in rRNA gene transcription (Lafarga *et al*., 2017). The inactivated rDNA genes are preferably located in the central area of the FC while the activated rDNA genes are arranged in the periphery and extend into the DFC, where transcription occurs (Raska *et al*., 2006; Boisvert *et al*., 2007). Several studies show that the number and organization of the FCs in neurons correlate with the transcriptional activity of the rDNA (Berciano *et al*., 2007; Hernández-Verdún *et al*., 2010).

On the other hand, the DFC is constituted by fibrils in which the activated ribosomal genes are arranged. The nucleolus is enriched in components of the transcription machinery (RNA polymerase I and nucleolin), snoRNPs enriched in fibrillarin and gene products (Dragon *et al*., 2002; Mongelard and Bouvet, 2006; Sirri *et al*., 2008). Finally, in the GC the assembly of the subunits of the ribosomes is completed, and they are stored there until the export to the cytoplasm (Raska *et al*., 2006).

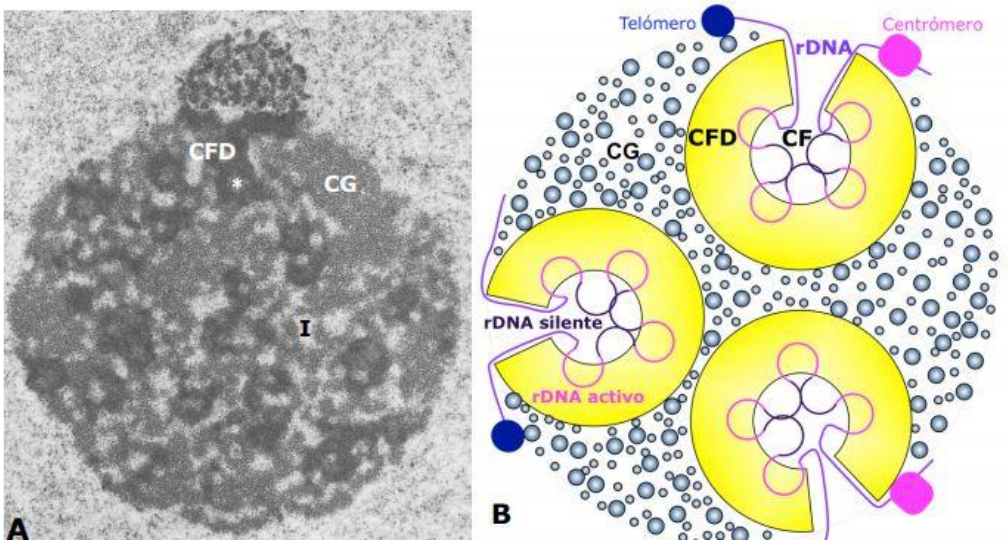


Figure 14. Nucleolus compartments. A: Ultrastructural image of a nucleolus belonging to a sensitive type A neuron. The three canonical subcompartments of the nucleolus are identified, small and numerous fibrillary centers (asterisk), the dense fibrillar component (DFC), the granular component (GC) and the interstices (I). **B**: Scheme of a nucleolus representing the three characteristic subcompartments of the nucleolus. The violet line corresponds to the rDNA that is introduced in the FC (silent rDNA, in dark) and partially organized on the inner edge of the DFC (transcriptionally active rDNA, fuchsia). In the GC the gray granules represent the large and small ribosomal particles (Taken from Mata, 2016).

The morphology and size of nucleoli are linked to nucleolar activity, which are inevitably altered under stress conditions, showing a variety of reorganization. The use of fluorescence-labeled antibodies against known markers of the nucleolus such as fibrillarin or UBF could reveal their distribution under nucleolar stress conditions. Typically, some stress conditions and diverse pathologies induce the redistribution of the nucleolar marker proteins, that start to aggregate in different regions and migrate towards the nucleolar periphery of the nucleolus (Shav-Tal *et al*., 2005). These kinds of morphological alterations have been designated as 'nucleolar segregation' to reflect a state of loss of nucleolar integrity. This segregation is characterized by the condensation and subsequent separation of the FC and GC (Boulon *et al*., 2010).

Nucleolar segregation has emerged as an indicator of nucleolar stress induced by agents that cause rDNA damage and rRNA transcription impairment (Calvo *et al*., 2012). For instance, chemotherapeutic agents that inhibit rRNA transcription and early processing steps, lead to the loss of nucleolar integrity (Burger *et al*., 2010).

The shape and size of the nucleolus are frequently altered in disease situations. These morphological differences are often correlated with both quantitative and qualitative differences in ribosome synthesis. In addition, defective ribosome synthesis recently emerged as a possible causal effect for several human diseases with the suggestion that accumulation of chemically modified ribosomes, e.g., oxidized particles, might contribute to the progression of neurodegenerative diseases such as Alzheimer and Parkinson diseases (Lafontaine, 2010).

Linking neurodegenerative diseases to nucleolar stress is an emerging field with tremendous potential in revealing molecular pathologies and eventual therapeutic strategies. Neurodegenarative diseases associated with nucleolar stress include Parkinson disease (Parlato *et al*., 2014) trinucleotide repeat (polyglutamine) disorders such as spinocerebellar ataxias and Huntington's disease (Tsoi *et al*., 2013; 2014), Alzheimer disease *(*Pietrzak *et al*., 2011*)* and amyotrophic lateral sclerosis (ALS) (Li *et al*., 2010).

In addition, recently, chemotherapeutic agents were discriminated for their effects on nucleolar morphology, prerRNA synthesis, and pre-rRNA

processing; strikingly drugs that affect most strongly RNA synthesis and early steps of ribosome synthesis (actinomycin D, cisplatin, and roscovitine) are those that most markedly alter nucleolar morphology while those that affect later stages of ribosome assembly leave the nucleolus relatively intact (Burger *et al*., 2010).

3.2. The Cajal body

The Cajal body (CB) is a multifunctional nuclear organelle present in all the eukaryotes. CBs are intimately linked with the nucleolus, both on physical and functional levels and were originally called ‘‘nucleolar accessory bodies’’ because of their close association with nucleolus in neurons (Gall, 2000; Lafarga *et al*., 2009). Its functions include the involvement in the biogenesis and maturation of the snRNPs and snoRNPs, required for pre-mRNA and pre-rRNA processing. snoRNPs, which are subsequently transported to the nucleoli, where they participate in rRNA processing. (Nizami *et al*., 2010; Machyna *et al*., 2013). The biogenesis of snRNPs is a complex process that includes both cytoplasmic and nuclear stages in which the SMN complex (formed by the motor neuron survival protein (SMN) and a protein family called Gemins) plays a key role (Li *et al*., 2014). The SMN complex acts as a chaperone, coupling the two components of the ribonucleoproteins (the Sm complex and the small nuclear RNAs, UsnRNAs). The CB is key in the final stage of the maturation of the pre-snRNPs. For this purpose, it has the structural protein coilin and scaRNAs (Xu *et al*., 2005). In general, both nucleoli and CBs are also involved in the production of non-poly(A)-tailed RNAs that are tightly connected to cell growth, including histone mRNAs, snRNAs, and snoRNAs in CBs, and rRNAs in nucleoli. Consistent with this, both structures are prominent in cells that are transcriptionally and metabolically active, such as neuronal and cancer cells (Berciano *et al*., 2007). Therefore, the number of CBs positively correlates with neuronal global transcriptional activity (Berciano *et al*., 2007; Baltanás *et al*., 2011; Machyna *et al*., 2013; Parlato and Kreiner, 2013).

Altogether, these findings suggest an intimate link between CBs and nucleoli. Given that the nucleolus acts as a major hub in coordinating the stress response, it is not surprising that the related CBs are also involved in the

cellular response to stress. There is little current literature on the contribution of CBs to the molecular pathophysiology of neurodegenerative disorders. For example, gene silencing in Purkinje cells clearly correlates with progressive disruption of both nucleoli and CBs (Baltanás *et al*., 2011). An important point is the contribution of CB dysfunction to the molecular pathophysiology of 2 motor neuron diseases: SMA and ALS. This disease is caused by deletion or mutation of the survival motor neuron 1 (*SMN1*) gene, resulting in reduced levels of SMN with diminished snRNP activity and the disruption of CBs. The depletion of canonical CBs seems to be a hallmark feature of SMA motor neuron (Tapia *et al*., 2017).

Specific objetives

In the present Thesis, we proposed the following specific objectives:

1. To define the changes in the pattern of global DNA methylation induced by sciatic nerve injury in neurons of dorsal root ganglia and spinal dorsal horn, and the consequences of miR-30c-5p-gain and -loss-of-function.

2. To determine the expression of DNA methyltransferases (DNMTs) in the dorsal root ganglia and the spinal dorsal horn after sciatic nerve injury and its modulation by miR-30c-5p targeting.

3. To validate in vitro DNMT3a and DNMT3b as targets for miR-30c-5p in cultured cell lines.

4. To assess the relationship between the methylation state of nociception-relevant-genes in the spinal dorsal horn and the intensity of neuropathic pain after sciatic nerve injury.

5. To define the changes of H3K9me3 induced by sciatic nerve injury in neurons of dorsal root ganglia and spinal dorsal horn, and the consequences of miR-30c-5p targeting.

6. To determine the expression levels of Suv39h1 in the dorsal root ganglia and the spinal dorsal horn after sciatic nerve injury and its modulation by miR-30c-5p targeting.

7. To assess in DRG neurons the morphological and ultrastructural consequences of miR-30c-5p-gain and -loss-of-function after sciatic nerve injury. Specifically, we will focus on the protein synthesis machinery: the Nissl bodies, and the nucleolus and its functional partner, the Cajal body.

Material and methods

1. Animals

For the experimental procedures of the present doctoral thesis, biological material from experimental animals was used. The experiments were performed in 8- to 12-week-old (250-300g) male Sprague-Dawley rats, group-housed (2-3 rats per cage). All animals were kept under standard controlled condition (22 ± 1ºC, 60-70% relative humidity), on a 12 h light/dark cycle (lights on at 8:00 a.m.). Food and water were supplied *ad libitum*. The study was approved by the Committee of Laboratory Animal Care and Use of the University of Cantabria (reference IP0415) and conducted in accordance with the guidelines from directive 2010/63/EU of the European Parliament and the International Association for the Study of Pain (Zimmermann, 1983). All animals received humane care and all efforts were made to minimize the number of animals used and their suffering.

1.1 Experimental model of neuropathic pain

1.1.1 Traumatic peripheral neuropathy model: Spared Nerve Injury

The animal model of traumatic neuropathy chosen to cause chronic neuropathic pain in rats was described by Decosterd and Woolf in 2000. Rats were anesthetized with isoflurane (Forane ®, 1.5-2.5%, 30% N_20 and 70 % O_2). An incision was made in the skin of the left paw thigh approximately 2 cm at the level of the femur and parallel to it. The femoral muscle was dissected, and the sciatic nerve was exposed at the level of its trifurcation of its three branches; sural, tibial and peroneal. In this model of chronic neuropathic pain, two ligatures of the tibial and peroneal branches were performed with suture, one in the area closest to the trifurcation and another more distal separated 2-3 mm. Subsequently, the axotomy of both branches was performed, leaving the sural branch intact (**Fig. 15**). Special care was taken to avoid contact or stretching of the sural branch. Once the nerve injury was performed, the muscles and skin were sutured with Vicryl® 5-0s suture (Ethicon, Johnson and Johnson). In control animals (sham) the left sciatic nerve was exposed following the same procedure, but it was left intact without any lesion.

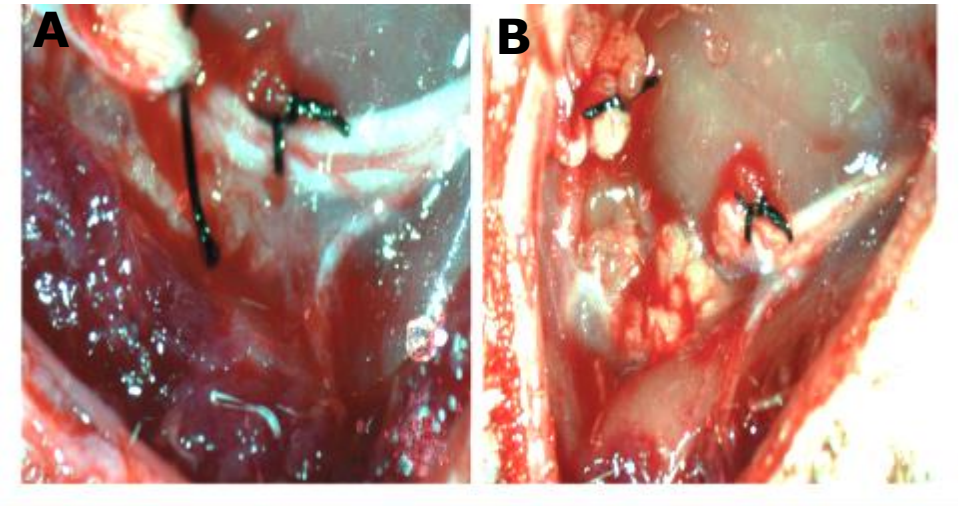


Figure 15. Representative images of the surgical intervention to cause the traumatic injury. A: The sciatic nerve is shown with its three branches: tibial, peroneal (ligated) and sural (free) before the injury and after the axotomy of the tibial and peroneal branches **(B).** The sural branch remained intact.

1.1.2 Assessment of the response to mechanical stimuli: Von Frey test

The degree of mechanical hyperalgesia and allodynia was measured using von Frey test. The animals were placed individually in a grid floor cage, where their mobility was reduced to movements on themselves. After a period of adaptation of 10 minutes to their new habitat, the plantar surface of the paw was stimulated with a series of von Frey monofilaments (Semmes Weinstein von Frey Aesthesiometer for Touch Assessment, Stoelting Co, Illinois USA) graduated on a scale based on the pressure in grams that they produce (**Fig. 16**). The shaking, licking or withdrawal of the paw after applying the mechanical stimulus was considered a positive response. The paws were stimulated 5 times with each of the monofilaments and the percentage of positive responses was evaluated. Initially, the paw was stimulated with a monofilament of intermediate strength and monofilaments of progressively decreasing force were used after until no response was obtained. Subsequently, stimuli of ascending force were applied, starting from the monofilament consecutive to the initial, until 100% of responses were provoked (Bonin *et al*., 2014)

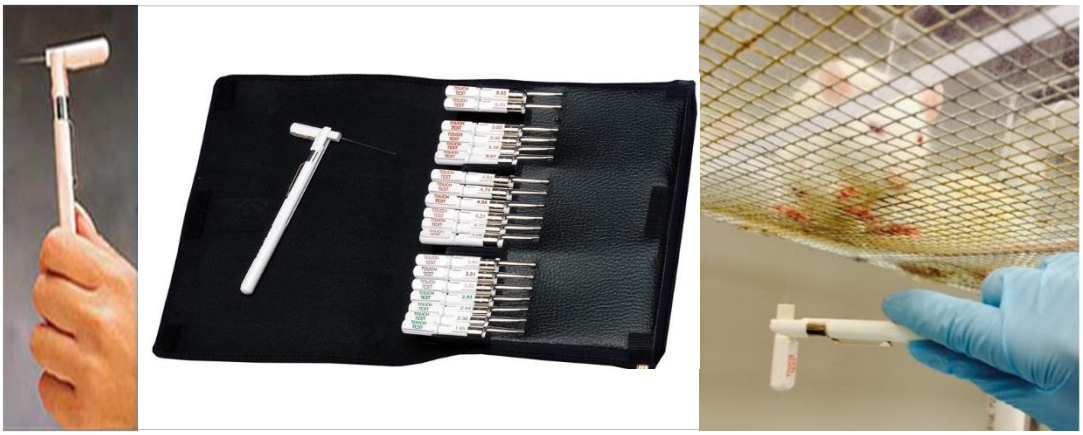


Figure 16. Von Frey monofilament set used to induce mechanical stimuli in the plantar surface of the hind paws of the animals.

The animals with neuropathy presented hyperalgesia and allodynia to mechanical stimuli in the area innervated by the sural branch (external zone of the paw, corresponding to the 4th and 5th fingers). The degree of mechanical allodynia developed was performed the day before the surgery (basal) and every day after surgery until the moment of sacrifice. The neuropathy became evident from the 7^{th} day after the surgery, being the 10^{th} day the high point, in terms of the severity of allodynia.

1.2 Treatments and experimental groups

The effects of treatment with miR-30c-5p and its antagonism on nociceptive perception were determined in the SNI rat model of chronic neuropathic pain. All the experimental groups were constituted by a minimum of 5-8 rats. The treatments were administered in the cisterna magna (alternate day injections) dissolved in lipofectamine (Life Technologies, Invitrogen) and artificial cerebrospinal fluid. Its composition was a mixture (1:1) of Solution A and B:

- **Solution A (in 5 ml of distilled H_20)**
  NaCl..... 0.0866 g
  KCl..... 0.00224 g
  $CaCl_2$+ $2H_2O$ .... 0.00206 g
  $MgCl_2$+ $6H_2O$ .... 0.00163 g

- **Solution B (in 5 ml of distilled H_20)**
  Na_2HPO_4 + $7H_2O$ 0.00214 g
  NaH_2PO_4 + H_2O .. 0.00027 g

The rats were anesthetized with isoflurane (Florane®, 11/min at 2.5-3%), and placed in a stereotaxic instrument, with the body in horizontal position and the head inclined 45°C with respect to the body (**Fig. 17**). The occipital area was depilated and disinfected with 70% ethanol. A 25G needle connected to a Hamilton syringe of 10 µl was introduced in the midline of the occipital crest, on a palpable surface between the occipital protuberances and the atlas.

Figure 17. Rat placed in the stereotaxic instrument were treatment administration or LCR extraction took place.

miR-30c-5p mimic (mirVana® miR-mimic, Life Technologies, UGUAAACAUCCUACACUCUCAGC) and miR-30c-5p inhibitor (mirVana® miR-inhibitor, Life Technologies) dissolved in lipofectamine were administered at a dose of 100 ng in a volume of 10 µl. The following experimental protocols were used (**Fig. 18A** and **B**). In a first experimental protocol, rats received a cycle of three intracisternal injections of miR-30c-5p mimic at the time of the sciatic nerve injury or sham intervention and on days 2 and 4 after surgery (**Fig. 18A**). In a second experimental protocol, rats received a cycle of three intracisternal injections of miR-30c-5p inhibitor at the time of the sciatic nerve injury or sham intervention and on days 4 and 7 after surgery (**Fig. 18B**). Rats were sacrifice when maximal differences were observed in the degree of mechanical allodynia developed between groups, on day 5 and 10, respectively. Sham animals received intracisternal injections composed of artificial CSF (9.26 µl) and lipofectamine (0.74 µl) following identical protocols.

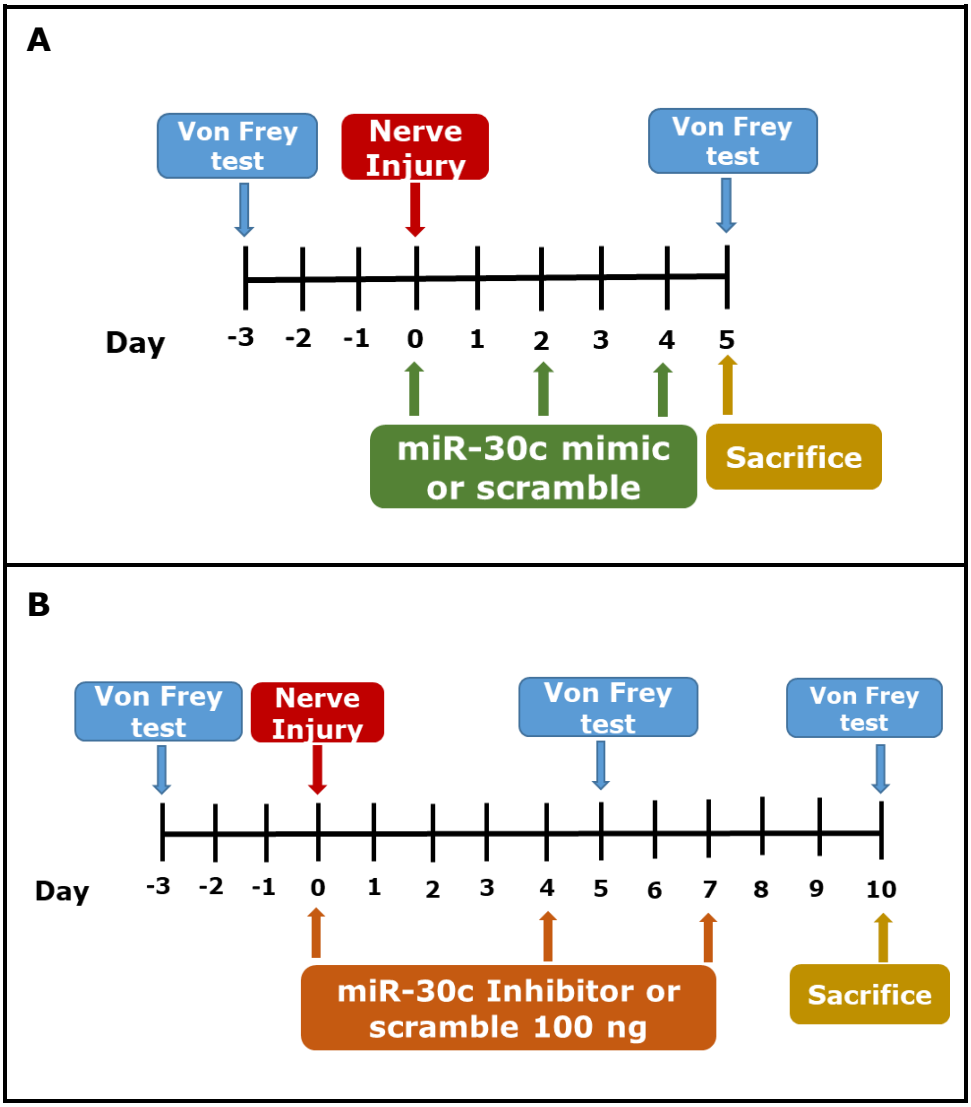


Figure 18. Experimental protocol of chronic treatment. Animals were administered the day of the surgery and on day 2 and 4 after the surgery with 100 ng of miR-30c-5p mimic (**A**) or the day of the surgery and on day 2 and 7 after the surgery with 100 ng of miR-30c-5p inhibitor (**B**). The effect of the treatments was evaluated with the von Frey test every other day after the nerve injury until the moment of sacrifice.

1.3 Processing of biological material

1.3.1 Spinal cord and dorsal root ganglion extraction

The animals were decapitated under anesthesia with 2% isoflurane. The spinal cords were extracted by hydroextrusion. After the decapitation of the animal, a cross section of the vertebral column was performed at the height of the iliac crest. A needle connected to a 15 ml syringe containing physiological saline (0.9% NaCl) was inserted into the distal orifice of the vertebral canal and pressure was exerted until the spinal cord exited through

the proximal orifice. Spinal cords were dissected by the midline to separate the two hemi spinal cords (ipsilateral and contralateral) from which the lumbar region was extracted. After spinal cord extraction, L3, L4 and L5 DRGs were dissected using small sharp forceps. Special care was taken to remove any fibrous structures surrounding DRGs and to cut off any attached nerve roots. Samples were then frozen in dry ice and stored at -80 °C until further use.

1.3.2 Perfusion technique

The animals were deeply anesthetized using 2,2,2-Tribromoethanol (Aldrich) administered intraperitoneally at a dose of 25 mg/kg. Once the total absence of reflexed movements was verified, the tissues were fixed by transcardiac perfusion according to the procedure described by Palay and Chan-Palay (1974). An incision was made in Y shape with an abdominal central section and two lateral sections following the costal margin. Subsequently, the thoracic cavity was opened, sectioning the diaphragm in its anterior insertion, and the breastplate was lifted by pinching the lower part of the sternum, thus exposing the heart. After that, a Teflon catheter Abbocath-T® was introduced through the apex of the left ventricle to the origin of the aortic arch to channel the entrance of the fixing solution containing 3.7% formaldehyde (freshly prepared from paraformaldehyde) in phosphate buffered saline (PBS). Then, an incision was made in the right atrium of the heart to create an open circulation and thus allow the exit of the solution. To propel the fixing solution, a Masterflex® perfusion pump was used at a flow rate of approximately 25 ml/min and approximately the same volume (in ml) of fixing solution as grams of weight of the animal was pumped. After the fixation, with the help of scissors and thin tweezers, the spinal cord and the dorsal root ganglion were obtained carefully and stored in PBS at 4°C until its further use in different histological techniques.

1.3.3 Obtaining neuronal dissociates

Neuronal dissociates from the DRGs and SDH were used for the immunofluorescence studies. To carry out this technique, we used the tissue fixed by perfusion with 3.7% paraformaldehyde (Merk) in PBS (PBS 1x: 137 mM NaCl;2.7 mM KCl; 10 mM Na_2HPO_4; 1.75 mM KH_2PO_4; pH 7.4). Squash

or neuronal dissociation technique consists of depositing on a siliconized slide (SuperFrostPlus, Menzel-Gläser, Germany) a small piece of tissue obtained by microdissection of the peripheral region of the dorsal root ganglion or the dorsal horn of the spinal cord. The sample is then covered in a 10 µl drop of PBS and an 18x18 mm coverslip is placed above it. Then, with the help of a histological needle, the neurons are dissociated by percussion. The degree of dissociation and the cytological preservation of the neurons was controlled under a phase-contrast microscope. Samples were frozen on dry ice for 5 minutes to ensure that the neurons adhered to the slide and then the coverslide was easily removed with a blade.

Subsequently, samples were stabilized by immersing the slides in 96% ethanol at 4°C for 7 minutes. Slides were stored in PBS at 4°C until its further use. The neuronal dissociates obtained through this methodology, which allows to have hundreds of perfectly preserved neural bodies, in combination with the help of a confocal microscope (Zeiss, LSM 510) have been fundamental for the development of this Doctoral thesis. Thus, optical sections of the entire neuronal soma can be made in those preparations; this allows to determine with high resolution the distribution of the molecules under study in the nuclear compartments and to establish, in addition, the possible colocalization of molecules in those compartments. In addition, squash is also very useful to perform quantitative analysis with high reliability since in the digitized projection of all sections, structures considered of interest for the study can be quantified.

2. Microscopy techniques

2.1 Confocal laser microscopy: Immunofluorescence

For conventional immunofluorescence, the dissociated neurons of the SDH and DRG obtained by the squash technique were incubated 20 min with glycine 0.1M to remove residual aldehyde groups after fixation. After that, the samples were permeabilized with PBS-Triton X-100 0.5% for 45 min at room temperature (RT) in light agitation, and washed in PBS-0.05% Tween-20. We proceeded with the indirect immunofluorescence labeling with a primary antibody diluted in PBS-BSA 1%. For this purpose, the dissociated area on the slide was delimited with a diamond-tipped pencil and 10 µl of the

primary antibody were added per preparation and incubated overnight in a humid chamber at 4°C in the dark. After several washes in PBS-T, the slides were incubated with a secondary antibody conjugated with a fluorochrome, fluorescein (FITC), Cy3 or Cy5 (Jackson ImmunoResearch Laboratories, Inc.,West Grove, Pennsylvania, USA) for 45 min in a dark and humid chamber at RT. Some slides were contrasted with a solution of propidium iodide (PI) (dilution 1:2000 from a stock of 1 mg/ml), which specifically dyes the nucleic acids and, in neurons, contrasts very well the nucleolus and Nissl bodies.

Samples were mounted with VectaShield medium (Vector Laboratorioes, Peterborough), sealed with nail lacquer and then examined with a Zeiss LSM 510 laser confocal microscope with three laser lines; argon (488 nm), HeNe (543 nm) and HeNe (633 nm), to excite FITC, TxRd and Cy3 respectively. The specific antibodies used in this study appear listed in **Table 1**.

Antibody	Marker	Type	Reference	Dilution
5'-MethylCitosine	Methylated DNA	Mouse Monoclonal	Eurogentec(091105)	IF:1/100
UBF	Upstream Binding Factor	Mouse Monoclonal	Santa Cruz	IF:1/100
Coilin	Cajal bodies	Rabbit polyclonal	Bohmann *et al*.,1995	IF:1/300
DNMT3b	DNA methyltransferase 3b	Rabbit monoclonal	Abcam 2851	WB: 1/500
DNMT3a	DNA methyltransferase 3a	Rabbit monoclonal	Abcam 2851	WB:1/1000
Lamb1	Nuclear lamina B1	Rabbit monoclonal		IF:1/100 WB: 1/1000
H3K9me3	Histone H3 Lys9 trimethylation			

Table1. List of antibodies used for immunofluorescence and western blot

2.1.1 5'-Methylcitosine detection

The dissociated neurons from the SDH and DRG were used for the detection of 5'-methylcitosine (5'-MeC) by confocal laser microscopy according to the previously described protocol. To carry out this technique the tissue was fixed by perfusion with PFA at 3.7% in HEPEM (HEPEM 1x: 130 mM Pipes disodium salt, 60 mM HEPES, 4 mM $MgCl_2 \cdot 6H_2O$; 20 mM EGTA; pH 6.9) with Triton X-100 0.5%. The fixed material was washed in HEPEM 1x and stored in PBS at 4ºC until further processing. Once the squashes were obtained as described above, the DNA was denatured with HCl 4N + 0.1% Triton X-100 for 20 minutes at room temperature. To neutralize the action of HCl, 6 washes of 6 minutes were performed in 50 mM Tris pH 8. After that, samples were incubated in Tris 50 mM pH 8 with 1% BSA for 2 hours at 4°C. Finally, 3 washes of 5 minutes in Triton X-100 0.5% were performed, after which the primary antibody anti 5'-MeC (Mouse, Eurogentec) was incubated and continued as if it were a conventional immunofluorescence, as it is indicated in the previous section.

2.2 Conventional transmission electron microscopy

For the study of transmission electron microcopy, we used the technique described by Palay and Chan-Palay (1974). After the perfusion of the animals with 3% glutaraldehyde in 0.12M phosphate buffer (pH 7.4) the SDH and DRG were extracted and post-fixed in the same solution for approximately 1 hour at RT. After several washes of 15 minutes in 0.12M phosphate buffer, the microdissected DRG and SDH were post-fixed in 2% osmium tetroxide solution for 2 hours at room temperature and protected from light. Before proceeding with the dehydration, the material was washed several times under agitation with a saline solution (2.4% NaCl). For the process of dehydration, we followed the next steps:

Acetone 30%, 15 minutes

Acetone 50%, 15 minutes

Acetone 70% + uranyl acetate 1% 30 minutes

Acetone 80%, 30 minutes

Acetone 90%, 30 minutes

Acetone anhydrous, 30 minutes (two steps)

Propylene oxide, 30 minutes (two steps

The blocks with the tissues were included in araldite (Durcapan) following the indications specified by the manufacturer (ACM, Fluka AG, Switzerland) and they were introduced in a stove at 65°C for 48 hours for polymerization. Finally, the blocks were cut with an ultramicrotome (Leica, Ultracut UCT). Semi-fine slices of 1 µm thickness were made, which were stained with toluidine blue 1% to assess the correct preservation of the material and to select the area of the SDH and DRG that we wanted to use for ultramicrotomy. The ultrathin sections of 50 nm were mounted on grids covered with a Formvar film (SigmaAldrich) and then stained with uranyl acetate and lead citrate according to Reynolds (1963) for observation with an electron microscope (Philips EM 208). Images were taken in an optical microscope (AxiosKop2 plus) coupled to a video camera (AxioCam HRC Zeiss), to later attach them to an image analyzer.

3. Molecular biology techniques

3.1 Protein analysis by western blot

Rats were sacrificed by decapitation with a guillotine and the DRGs and SDH were extracted and frozen at -80°C until the moment of use. For the protein extraction, 100 µL of NETN lysis buffer were added for each 10 mg of DRG or SHD tissue. NETN buffer was used from the stock (200 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA), 500 mM final concentration of NaCl, Benzonase (1µl/ml, Novagen) and protease inhibitors (1:100, Roche). Samples were homogenized with a polytron and incubated on ice for 30 min. Subsequently, the samples were centrifuged at 14.000 rpm for 10 minutes at 4°C and the supernatant was collected in a sterile tube. Finally, the protein concentration was measured using the Lowry method (Sengupta and Chattopadyay, 1993) in the spectrophotometer (Multiskan ex, Thermo Scientific) at a wavelength of 620 nm, using a standard curve of albumin. Samples were stored at -20°C until later use.

Samples were heated at 95°C for 5 minutes in loading buffer (2X buffer: 60 mM Tris-HCl pH 6.8, 2% SDS; 10% glycerol; 0.02% bromophenol blue) and

centrifuged at 3000 rpm for 90 s. Equal amounts of proteins (50 ng) were subjected to electrophoresis on 10% gradient gels, which composition is shown in **Table 2**.

Components	Running Gel	Stacking Gel
Destiled H_20	4.65 ml	1.5 ml
30% Acrilamide-Bisacrilamide	3.6 ml	451 µl
Running Buffer 4X	2.85 ml	--
Stacking Buffer 4X	--	670 µl
Amonium Persulfate (APS)	150 µl	42 µl
Temed	7.5 µl	5 µl

Table 2. Components of 10% acrylamide gel for western blot

10 µl of a standard protein marker was used as a reference for the molecular weights. After loading the samples, gel run for 15 min at current of 100 V and for 90 min at 130 V in running buffer. Following the electrophoresis, samples were transfected to a polyvinylidene difluoride (PVDF) membrane on transfer buffer subjected to a continuous current of 100 V for 90 min at 4°C. The efficiency of the transfer was checked by staining the membrane with Ponceau red. The membranes were washed with TBS-T until the dye disappeared. Then, the membranes were incubated for 2 hours in a blocking solution composed of a 3% BSA solution in TBS-T buffer (5M NaCl, Tris pH 7.6 1M). The blocking process prevents possible nonspecific binding of the antibody to the membrane. After removing the membranes from the blocking solution, they were incubated with the primary antibody diluted in TBS-T, overnight at 4°C under agitation. The primary antibodies used in this section appear listed in **Table 1**. After incubation with the primary antibody, the membranes were washed 2 times in TBS-T for 10 minutes at room temperature. Then, the membranes were incubated with the corresponding secondary antibody (1:10000 in TBS-T and 2% of BSA) for 30 minutes, under agitation at room temperature. Next, the membranes were washed in TBST (6 x 10 min). The following secondary antibodies were used: IRDyeTM 680/800 anti-mouse IgG (1:10000, Rockland) and IRDyeTM 680/800 anti-IgG rabbit (1:10000, Rockland) that were detected using the Odyssey Infrared Imaging System (Li-Cor) system.

3.2 mRNA and miRNAS expression by quantitative PCR

3.2.1 mRNA and miRNA extraction in SDH and DRG

Rats were sacrificed by decapitation with a guillotine and the DRGs and SDH were extracted and frozen at -80°C. RNA was extracted following the Trizol protocol. Trizol (1 ml/mg tissue) was added to the samples and they were homogenized with a polytron. They were shaken for 15 s and incubated for 3 min at RT. 0.2 volumes of chloroform per volume of Trizol were added, vortexed for 15 s and incubated for 3 min at RT. Then, they were centrifuged at 12.000 rpm for 15 min at 4°C. The supernatant aqueous phase (approximately 400 µl) was collected and 500 µl of isopropanol/ml Trizol were added. The mixture was stirred for 5 s and incubated for 30 min on ice. Samples were centrifuged at 12.000 rpm for 10 minutes at 4°C. Supernatant was carefully discarded and 1 ml of ethanol 70% for each ml of Trizol used was added. Then, it was centrifuged for 5 min at 7600 rpm. Supernatant was discarded and the pellet was allowed to dry. The pellet was resuspended in 20 µl of depc H_2O. The quantification of the samples was done by reading their absorbance at 260 nm in a spectrophotometer (Nanodrop 1000V 3.6, Thermo scientific Inc). Finally, the purity was measured by the quotient between absorbance at 260 nm (nucleic acids absorbation) and 280 nm, which refers to the amount of proteins present in the sample. A result of 2.0 indicates a pure RNA, without contaminants. In all the experiments, this quotient was always higher than 1.8.

3.2.2 mRNA and miRNA reverse transcription

From the purified RNAs obtained following the previously described protocol, cDNA was obtained by retrotranscription using a commercial RT-PCR kit (Revert-aid™ Minus First Strand cDNA synthesis kit, Fermentas). The reaction of reverse transcription of RNA (RT-PCR) and miRNA (microRT-PCR) is the method by which the complementary DNA or cDNA chains are obtained from the extracted RNA. For the reaction, 1 µg of RNA was used, to which 1 µl of generic primers and the amount of H_2O depc necessary to reach 12 µl were used. This first mixture was incubated at 70°C for 5 min. Subsequently, 4 µl of reaction buffer 5X, 1 µl of RNAse inhibiting enzyme (Ribolock Ribonuclease inhibitor) and 2 µl of a 10 mM solution of deoxynucleotide triphosphates

(dNTPs) were added. The mixture was incubated 5 min at RT and 1 µl of reverse transcriptase enzyme, responsible for the retrotranscription process, was added. The samples were placed in a thermocycler (MyCycler, Bio-Rad Laboratories Inc) programed with the following reaction conditions: 10 min at 25°C; 60 min at 42°C; 10 min at 70°C; a final cycle for the maintenance of the samples at 4°C. The obtained cDNA was kept at -20°C until its use.

For the reverse transcription of the miRNAs, a commercial RT-PCR kit was used. For the reaction, 100 ng of RNA were used and the amount of H_2O depc necessary to reach a final volume of 9.16 µl and 2.84 µl of a mixture containing; 0.15 µl of dNTPs, 1µl of the enzyme reverse transcriptase (multiscribe RT enzyme), 1.5 µl of reaction buffer 10X and 0.19 µl of RNAses inhibitors (Ribolock Ribonuclease Inhibitor). Finally, 3 µl of the specific primer corresponding to the miRNA to study were added to the mixture. The miRNAs used as internal controls were RNU6B (tissues) and cell-miR39 (fluids). The total sample (15 µl) was centrifuged and incubated on ice for 5 min. Samples were introduced in a thermocycler and the following conditions were used: 30 min at 16°C; 30 min at 42°C; 5 min at 85°C; a last cycle for the maintenance of the samples at 4°C. cDNA was stored at -20°C until its use.

3.2.3 Oligonucleotide primer design for quantitative PCR

For the design of the oligonucleotide primers, the online software "Oligo primer design" was used. The couples of oligonucleotides whose length was around 20 bases, their percentage of G+C was approximately 50%, its mT was ~60°C and its composition did not favor the appearance of secondary structures were considered appropriated. Furthermore, we checked using a nBLAST analysis (nucleotide Basic Local Alignment Seach Tool) that the amplicon product of the selected oligonucleotides would correspond only to the gene of interest and did not amplify other sequences in our animal model. The primers that have been used in this work to analyze the levels of gene expression by qPCR for SYBRGreen are shown in **Table 3**:

Name	Primer Forward/reverse (5'-3')
DNMT3B	Fw: GCAAGAGAGAGGCCCTCAG Rv.: TGTGAGGGAGATGCTCAGTG
DNMT3A	Fw: CCGGGTGCTATCTCTCTCTTTG Rv.: TGACGATGGAGAGGTCATTG
DNMT1	Fw: CGGCTCAAAGACTTGGAAAG Rv.: TAGCCAGGTAGCCTTCCTCA
Nfyc	Fw: CCTGTATCAGGCACCCAAGT Rv.: GGTGACTTGCTGGATCTGGT
Nfyc (ms-qPCR)	Fw: TGACCAATAAGGTGCCAGGT Rv.: CGCCATGTTGTGTCTTCG
TGFβ-1 (ms-qPCR)	Fw: GATCCTCCAGACAGCTAGGC Rv.: ACTCCTCCTCCCCCTCCT
Suv39h1	Fw: TAGCTGTTGGCTGTGAGTGC Rv.: CTGGCCTTGGTCATTGTAGG
18s	Fw: ACCGCAGCTAGGAATAAGGA Rv.: GCCTCAGTTCCGAAAACCA

Table 3. Primers used for quantitative PCR and ms-qPCR

3.2.4 Detection of gene expression by quantitative real time PCR

The expression levels of genes and miRNAs were determined by the quantitative polymerase chain reaction (PCR), which is one of the most sensitive methods for the quantification of gene expression from cDNA. The detection is done at the same time as the amplification, so this technology replaces traditional amplification and electrophoresis. The mRNA expression was normalized using 18S as the reference gene. The expression of the miRNas in tissue was normalized to RNU6B. Commercial TaqMan® probes (Applied Biosystems, Life Techonologies) and reagent containing Mg^{2+} and nucleotides (Premix Ex TaqTM Perfect Real Time, Takara Bio Inc) were use. The amplifications were made in a thermocycler for quantitative real-time PCR (Applied Biosystems 7500 v2.0.4, Life technologies) and MxPro analysis software(Stratagene). The reaction was carried out in duplicate, in a volume of 10 µl containing 0.5 µl of the µRT-PCR product, 5 µl of SYBRgreen Mix (Takara), 0.4 µl 10 µM of each sense and antisense primers corresponding to the gene of interest, 0.5 µl of the housekeeping gene and 3.75 µl of H_2O depc. The conditions of the qPCR that we have used are:

a) A first segment of denaturation
    I. I.1 cycle at 95°C for 30 seconds)
b) A ringing segment (40 cycles)
    I. 60°C for 30 seconds
    II. 72°C for 30 seconds.
c) The dissociation curve
    I. 1 cycle at 95ºC for 1 minute
    II. 1 cycle at 55ºC for 30 seconds
    III. 1 cycle at 95ºC for 30 seconds

Fluorescence measurements are taken at the end of each banding step of the second segment and in the final segment, to perform the dissociation curve, during the whole temperature rise from 55°C until 95°C. When the derivative of fluorescence is plotted against temperature, a peak corresponding to the melting temperature of the product (mT) appears. The peak area is proportional to the amount of amplification product obtained. Expression levels were determined in duplicated in two independent experiments. Results

are expressed as: $2^{(Ct\ reference-\ Ct\ problem)}$; being Ct the threshold cycle. Afterwards, all values were multiplied for a better data analysis. Values are expressed as the mean ± SEM of the relative expression obtained on the different experiments.

3.3 DNA isolation

Genomic DNA was isolated from the SDH of sham, SNI and SNI rats treated with either miR-30c-5p mimic or miR-30c-5p inhibitor using the NucleoSpin® Tissue (MN) kit. Approximately, 25 mg of tissue were lysed by incubation with proteinase K/SDS solution for 3 hours at 56°C. The samples were loaded in the NucleoSpin® Tissue Column and DNA was collected after several washes following the manufacturer's instructions. The quantification of the samples was done by reading their absorbance at 260 nm in a spectrophotometer (Nanodrop 1000V 3.6, Thermo scientific Inc). Finally, the purity was measured by the quotient between absorbance at 260 nm (nucleic acids absorbation) and 280 nm, which refers to the amount of proteins present in the sample. A result of 2.0 indicates a pure DNA, without contaminants. In all the experiments, this quotient was always higher than 1.8.

3.3.1 Methylation sensitive qPCR (ms-qPCR)

The methylation sensitive polymerase chain reaction (ms-qPCR) is a robust and reproducible method to rapidly profile the percentage of methylation status of numerous loci without the use of sodium bisulfite. This technique has three basic steps: (1) the digestion of a DNA sample of interest with several methylation sensitive restriction enzymes; (2) the designing of primers to specific genomic regions; (3) a real-time PCR amplification reaction to monitor the formation of the PCR product. (Oakes *et al*., 2016). To investigate Nfyc and TGF-β1 genes, 300 ng of DNA from SDH samples for each digestion reaction were digested with 10 units of the methylation sensitive enzymes FauI (BioLabs) or TauI (ThermoFisher)respectively or no enzyme for 2h at 55°C (temperature of activity of FauI), 600 rpm shaking. For amplification, primers were designed using Primer3Plus online software. Primer design involved the placement of primer pairs that flank both the region of interest and a control region. The control primers were designed to a region that was devoiced of any of the restriction sites of the enzymes used

in the design of the experiment. The other pair of primers were designed to flank specific regions based on the presence of informative restriction sites within the PCR amplified region. Primers were designed containing at least three CpG sited for the PCR amplification of both genes. Quantitative PCR was done using the SYBRgreen Mix (Takara). The undigested control represents 100% methylation because methylation-sensitive enzymes do not cut methylated DNA, allowing the entire sample to be amplified by PCR. The percentage of methylation was determined from the change in Ct value using the basic principle that each successive round of PCR amplification results in approximately a 2-fold increase in the amount of product. The relationship of ΔCt to percentage methylation was calculated using the formula $\%Me=100 (e^{-0.7(\Delta Ct)})$.

4. Cell culture studies

For this doctoral thesis, we chose to use the SH-SY5Y human neuroblastoma and HeLa cell lines (ATCC®: CRL-2266) to perform *in vitro* assays. Cells were cultured in 10 cm sterile Petri dishes (Iwaki®,Asahi Techno Glass Corporation) in Dulbecco's modified Eagle medium F12 (DMEM/F12 medium ,Gibco, referencia) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel), 100 U/ml penicillin (Gibco) and 100 U/ml streptomycin (Gibco) in a humidified incubator at 37°C and 5% CO_2. Every 2 days, media was aspirated and replaced with fresh media. Cells were split and passaged every 4-5 days at 80-90% confluence, following media aspiration and washing with 2 ml of PBS (Gibco). Next, PBS was aspirated and cells were trypsinized with 2 ml of 0.25% trypsin-EDTA (Gibco,Invitrogen) for 3 min, centrifuged at 1500 rpm for 5 minutes, resuspended in the appropriate amount of complete growth media and divided into individual tissue culture wares. Cells were subcultured 2-3 times per week at a 1:10 dilution in a new 10 cm petri dish.

For cryopreservation of cells, cells were trypsinized and pelleted as described above. After resuspension, cells were aliquoted into 2 ml cryogenic vials (IWAKI) and tissue culture grade dimethylsulphoxide (DMSO) (Sigma, Aldrich) was added to a final concentration of 10%. The cryovials were put in

an isopropanol cryobox at -80°C for 24 hours and finally transferred and stored at -80°C.

4.1 Dual luciferase reporter assay

Dual luciferase reporter assay is a useful tool to study gene expression at the transcriptional level. The use of a reporter gene such as luciferase is a reliable method to quantify the suppression of a target gene by a specific miRNA. These reporters contain the gene of interest coupled to a luciferase gene as a reporter gene. Using this system, any 3'UTR target site can be sub-cloned downstream of a reporter gene and co-transfected along with a specific miRNA into cells. The interaction between the miRNA and the miRNA target site will lead to reduction of the firefly luciferase expression. Subsequent inhibition of reporter gene expression by the miRNA can serve to validate the regulation of the gene. In this Doctoral Thesis, HeLa and SH-SY5Y cells were plated on 96-well opaque plate (Nunc, Denmark) at a density of 10.000 cells per well. 24 hours after plating, the growth medium was removed and replaced with Optimem (Gibco,Life Technologies). pMIR-3'UTR-DNMT3b (Promega, 25 nM) or pLightSwitch-3'UTR-DNMT3a (SwitchGear Genomics, 25 nM) were transfected into cells using X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich). Empty pMIR or pLightSwitch were used as a control. Twenty-four hours later, cells were transfected with miR-30c-5p mimic (10 nM) or scramble miRNA, according to the manufacturer's protocol. Twenty-four hours after the miRNA transfection, the cells were harvested and the activity firefly luciferase was measured using the luciferase reporter assay (Promega/SwitchGear Genomics). Relative luminescent units were normalized to total protein. The experiment was replicated three times.

5. Quantification and statistical analysis of relative gene expression

The statistical analysis was carried out with the statistical package GraphPad Prism 6 (GraphPad Inc, CA, USA). The values were expressed as the mean ± SEM of the final n of animals per group. Student's *t* test was used to evaluate the differences between two means of continuous variables. The data from the behavioral studies was compared by one-way ANOVA of repeated measures followed by Bonferroni test. For the comparison of gene expression

values between more than two groups, a one-way ANOVA followed by the Bonferroni test was used. Pearson's simple linear regression analysis was performed to correlate gene expression levels with each other. A p-value of less than 0.05 was considered statistically significant.

On the other hand, the squash technique allows to analyze the complete soma of neurons of the SDH and DRG. Thanks to this technique we quantified, i) the number of neurons with chromatolysis ii) the number of neurons with nuclear eccentricity iii) the proportion of neurons with nucleolar segregation iv) the mean intensity of the fluorescence of 5'-methylcitosine and H3K9me3 v) the percentage of 5'-MeC and H3K9me3 labeled area.

Results

1. Epigenetic changes in the somatosensory nervous system associated with neuropathic pain and its modulation by miRNA-30c-5p

1.1 The development of mechanical allodynia in rats is modulated in opposite ways by an miR-30c-5p mimic and an miR-30c-5p inhibitor

Previous results of our group support a major role for miR-30c-5p in neuropathic pain development (Tramullas *et al*., 2018). In this study, we show in rodent models of spared nerve injury that miR-30c-5p is up-regulated in the SDH, DRG, CSF and plasma, and that the expression of miR-30c-5p positively correlates with the severity of the allodynia developed after SNI. The administration of an miR-30c-5p inhibitor into the cisterna magna delays neuropathic pain development and reverses fully established allodynia in rats, whereas the treatment with miR-30c-5p mimic accelerates the development of allodynia. Moreover, in patients with neuropathic pain associated with leg ischemia, the expression of miR-30c-5p is increased in plasma and CSF compared to control patients without pain. Moreover, the expression levels of miR-30c-5p in plasma and CSF predict neuropathic pain occurrence in patients with chronic peripheral ischemia (Tramullas *et al*., 2018).

Our first objective in the present Thesis was to confirm and reproduce the nociception-related functional consequences of miR-30c-5p modulation *in vivo.* Neuropathic pain was induced in rats using the model of spared nerve injury of the sciatic nerve (SNI; n=8). Sham-operated animals served as controls (Sham; n=8). The development of allodynia to mechanical stimuli was evaluated in the ipsilateral hind paw of the animals using von Frey monofilaments of increasing strength. Nocifensive responses were assessed the day before SNI and, after surgery, daily for a follow-up period of 10 days. A series of rats subjected to SNI received a cycle of treatment with either miR-30c-5p inhibitor (SNI + miR-30c inhibitor; n=5) or miR-30c-5p mimic (SNI + miR-30c mimic; n=5), injected into the cisterna magna (100 ng/10µl). The first administration was at the time of nerve injury or sham interventions, and two more postoperative injections were administered on days 4 and 7 for

miR-30c-5p inhibitor (**Fig. 19A**), or on days 2 and 4, for miR-30c-5p mimic (**Fig. 19B**).

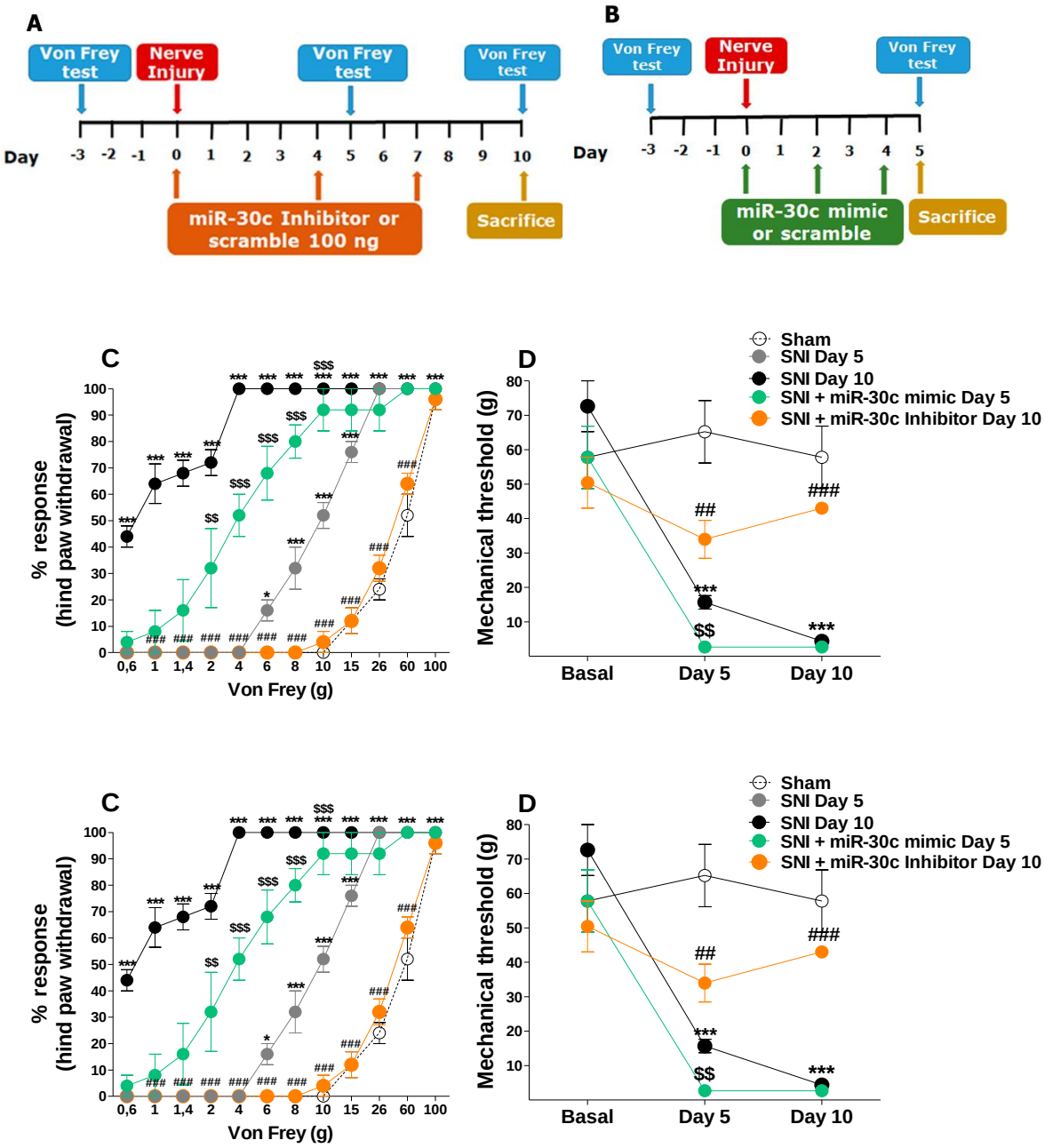


Figure 19. The development of mechanical allodynia after sciatic nerve injury in rats is prevented by miR-30c-5p inhibitor while accelerated by miR-30c-5p mimic. A: Rats subjected to spared nerve injury of the sciatic nerve (SNI) received a cycle of three intracisternal injections of miR-30c-5p inhibitor (100 ng/10µl; n = 5) or vehicle (n = 8). The first administration was at the time of sciatic nerve injury (SNI) and two more injections were administered on days 4 and 7 after surgery. Sham-rats (n = 8) served as controls. **B**: SNI-rats received a cycle of three intracisternal injections of miR-30c-5p mimic (100 ng/10µl; n = 5) or vehicle (n = 5) into the cisterna magna. The first administration was at the time of nerve injury and two more injections were administered on days 2 and 4 after surgery. **C**: The curves represent the percentage of hind paw withdrawal responses induced by von Frey

monofilaments of increasing force (g) in sham-and SNI-rats treated with vehicle, miR-30c-5p mimic or miR-30c-5p inhibitor, on days 5 and 10 after SNI. Rats that received miR-30c-5p inhibitor are protected against neuropathic pain development while rats treated with miR-30c-5p mimic significantly accelerate the development of mechanical allodynia; *p<0.05, **p<0.01, *** p<0.001 vs sham; $ p<0.05, $$ p<0.01, $$$ p<0.001 vs SNI Day 5; # p<0.05, ##p<0.01, ### p<0,001 vs SNI day 10(repeated-measures two-way ANOVA followed by the Bonferroni post hoc test). **D**: Threshold force (g) required to elicit responses 50% of the time at baseline and on days 5 and 10 after SNI or sham surgery (mean ± SEM). SNI-rats treated with miR-30c-5p mimic show lower mechanical thresholds than SNI-rats treated with vehicle on day 5 after SNI. SNI-rats treated with miR-30c-5p inhibitor do not develop allodynia during the 10-day follow-up period after SNI. *** p<0.001 vs sham; $$ p<0.01, ##p<0.01, ### p<0,001 vs SNI day 10 (repeated-measures two-way ANOVA followed by the Bonferroni post hoc test).

Our results confirmed that intracisternal administration of miR-30c-5p inhibitor prevents neuropathic pain development, whereas miR-30c-5p mimic accelerated the development of mechanical allodynia after SNI in rats. **Figure 19C** shows the development of neuropathic pain by SNI-rats, as indicated by the significant increases in their mechanical nocifensive responses to mechanical stimuli on days 5 and 10 after SNI compared with sham-rats responses (Two-way ANOVA; SNI day 5: "force intensity x nerve injury": $F_{(13,112)}$ =24.17, ***p<0.001; "nerve injury": $F_{(1,112)}$ =124.5, ***p<0.001; "force intensity": $F_{(13,112)}$ =537.6, ***p<0.001 vs Sham. SNI day 10: "force intensity x nerve injury": $F_{(13,112)}$ =80.82, ***p<0.001; "nerve injury": $F_{(1,112)}$ =944.6, ***p<0.001; "force intensity": $F_{(13,112)}$ =199.1, ***p<0.001 vs Sham). On the 10th day after nerve injury, SNI-rats treated with vehicle developed allodynia of maximal severity whereas the rats treated with miR-30c-5p inhibitor were free of pain (**Fig. 19D**) (Two-way ANOVA; "force intensity x nerve injury": $F_{(11,96)}$ =50.18, ###p<0.001; "nerve injury": $F_{(1,96)}$ =3267, ###p<0.001; "force intensity": $F_{(11,96)}$ =103.4, ###p<0.001 vs SNI Day 10). In contrast, the administration of miR-30c-5p mimic to SNI-rats accelerated the development of allodynia after the injury (Two-way ANOVA; "force intensity x nerve injury": $F_{(11, 96)}$ =6.089, $$$ p<0.001; "nerve injury": $F_{(1, 96)}$ =70.42, $$$ p<0.001; "force intensity": $F_{(11, 96)}$ =76.22, $$$ p<0.001 vs SNI Day 5). The animals were sacrificed when allodynia severity reached maximal differences with the SNI-animals (10 days for animals treated with miR-30c-5p inhibitor and 5 days for animals treated with miR-30c-5p mimic) (**Fig. 19A** and **19B**).

1.2 Dorsal root ganglion and spinal dorsal horn neurons present increased global levels of DNA methylation after sciatic nerve injury in rats

Increasing evidence points to the involvement of epigenetic processes in the altered expression of genes encoding ion channels, receptors, neurotransmitters, modulators, cytokines, etc., which underlie the pathological chronification of pain. Among them, aberrant DNA methylation contributes to cell-type specific variations in gene expression in different physiological and pathological processes, including chronic pain (Shao *et al., 2017;* Garriga *et al*., 2018). Therefore, the second objective of this Thesis was to provide insights on the contribution of DNA methylation to the establishment and long-term maintenance of neuropathic pain after the injury of a peripheral nerve. To this end, we analyzed the changes induced by SNI, on days 5 and 10 after the surgery, in the global DNA methylation levels as well as the distribution patterns in two pain-related areas, the SDH and DRG. Global DNA methylation was determined in isolated neurons (n=60; 20 neurons per animal and 3 rats per group) by immunofluorescence using an antibody to 5'methylcitosine (5'-MeC), which marks the methylated form of cytosine in the DNA.

Our results indicate that DRG neurons isolated from neuropathic rats showed a significant increase in both the average fluorescence intensity (One-way ANOVA: $F_{(2, 7)} = 31.58$, $p<0.01$) and the percentage of methylated area (One-way ANOVA: $F_{(2, 7)} = 13.76$, $p<0.01$) when compared to DRG from sham-rats (**Fig.20A** and **B**). 5'-MeC signal intensity was significantly higher in the DRG neurons isolated from 5 ($p<0.001$) and 10 ($p<0.05$) SNI-rats than in neurons isolated from sham-rats. However, 5'-MeC signal intensity decreased 10 days after the surgery when compared to 5 days SNI-animals ($p<0.05$). Moreover, DRG neurons from 10 days SNI-rats showed a higher percentage of methylated area when compared with DRG neurons from sham-rats ($p<0.05$).

5'-MeC immunostaining in the DRG neurons from sham-rats (**Fig. 20C)** was mainly detected in areas of heterochromatin, such as the perinuclear regions and the intranuclear clumps of heterochromatin. DRG neurons isolated from 5 and 10 days neuropathic rats showed that DNA methylation was

homogenously distributed though the nucleoplasm and, as expected, it was not detected in DNA lacking areas such as the speckles (**Fig. 20D and 20E**).

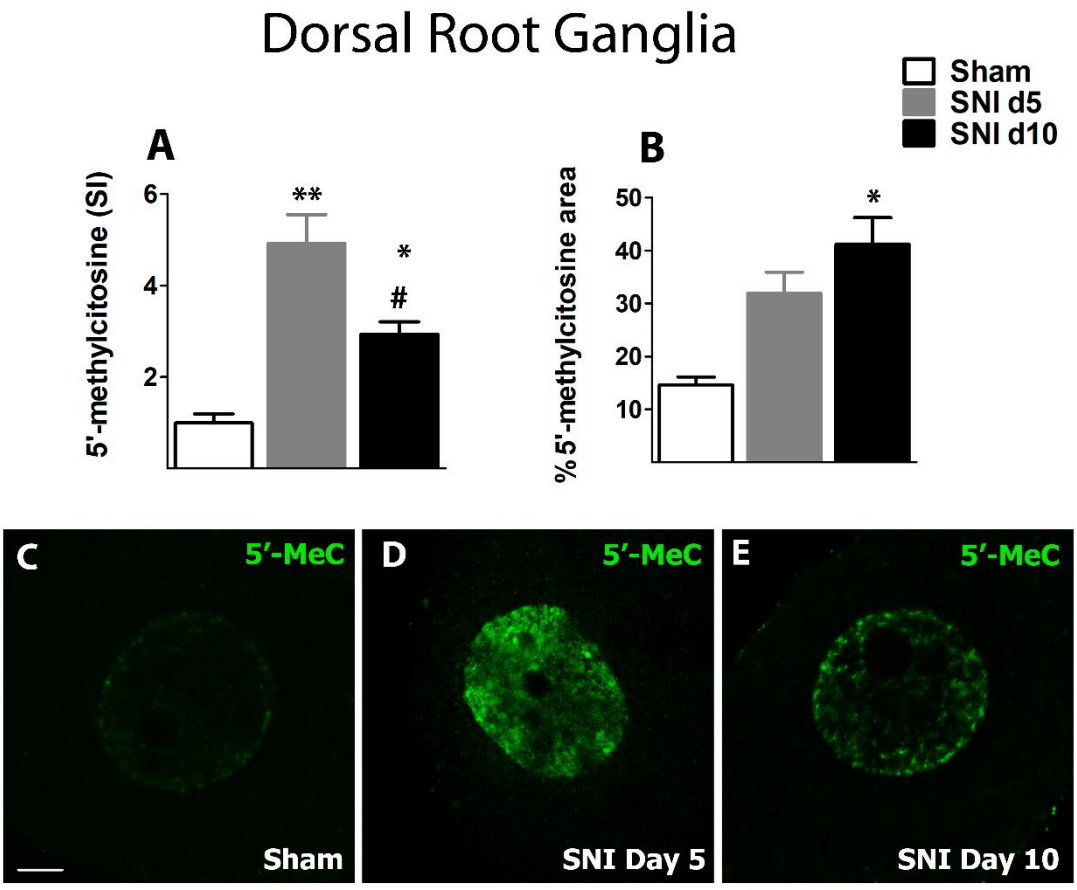


Figure 20. Cellular localization and distribution pattern of 5'methylcitosine in dorsal root ganglia neurons from sham, 5 days SNI rats and 10 days SNI rats. In dorsal root ganglia (DRG) neurons from sham rats (**C**), low levels of 5'methylcitosine-positive immunostaining are detected in areas of heterochromatin**.** In contrast, DRG from 5 (**D**) and 10 (**E**) days SNI rats present high fluorescence levels that were homogenously distributed through all the nucleoplasm. No positive signals were detected in DNA lacking areas such as the speckles. The averages of signal intensity (**A**) and percentage of methylated area (**B**) were determined in 20 neurons per rat (SNI: n=3; Sham: n= 3) * $p<0.05$, **$p<0.01$ vs Sham; # $p<0.05$, vs SNI Day 10(One-way ANOVA). Scale bar: 5 μm.

5'methylcitosine immunoreactivity was also higher in the nucleus of SDH sensory neurons isolated from 5 and 10 SNI-rats than in neurons isolated from sham-rats. As shown in **Fig. 21A** and **B**, the average fluorescence intensity (One-way ANOVA: $F_{(2, 7)} = 6.8$, $p<0.05$) and the percentage of methylated area (One-way ANOVA: $F_{(2, 7)} = 19.96$, $p<0.01$) were significantly higher in 5 (SI, $p<0.01$) and 10 days SNI- than in sham-rats and no differences were observed between 5 and 10 days SNI-rats in the 5'-MeC immunoreactivity.

The nucleus of sham neurons exhibited low immunoreactivity signals of 5'methylcitosine in heterochromatin areas and no signal was detected in DNA-free areas (**Fig. 21C**). SDH sensory neurons from SNI-rats showed higher levels of 5'-MeC immunoreactivity than sham-rats, with dense accumulations around the nuclear envelop and the nucleoplasm (**Fig. 21D** and **E**).

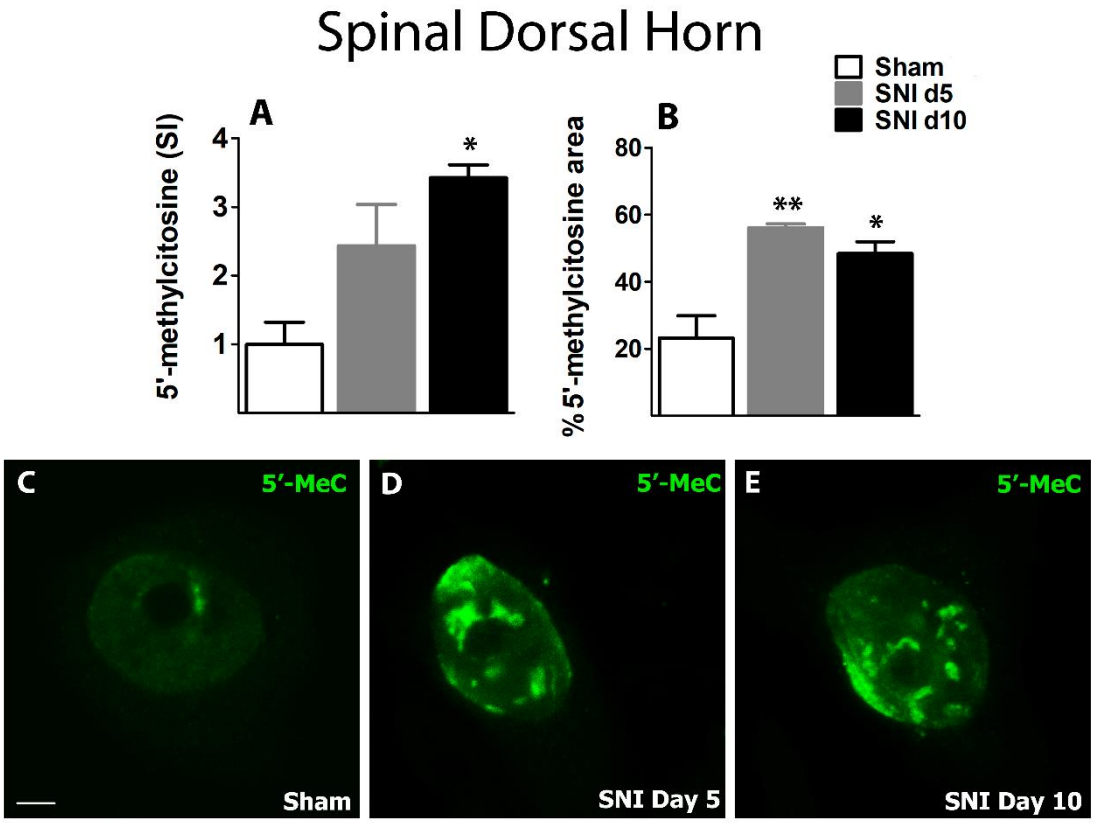


Figure 21. Cellular localization and distribution pattern of 5'methylcitosine in spinal dorsal horn neurons from sham, 5 days SNI-rats and 10 days SNI-rats. In spinal dorsal horn (SDH) neurons from sham rats (**C**), low levels of 5'-methylcitosine-positive immunostaining are detected in areas of heterochromatin**.** In contrast, SDH neurons from 5 (**D**) and 10 (**E**) days SNI rats present high fluorescence

levels homogenously distributed through all the nucleoplasm. No positive signals were detected in DNA lacking areas such as the speckles. The averages of signal intensity (**A**) and percentage of methylated area (**B**) were determined in 20 neurons per rat (SNI: n=3; Sham: n= 3) * $p<0.05$, **$p<0.01$ vs Sham; (One-way ANOVA). Scale bar: 5 µm.

These results indicate that neuropathic pain developed by rats after the traumatic injury of a peripheral nerve is associated with intense changes in DNA methylation in both the DRG nociceptors and the second order neurons (SDH) of the nociceptive pathway.

1.2.1 Dorsal root ganglion and spinal dorsal horn neurons present increased mRNA expression levels of DNMTs after sciatic nerve injury in rats

In mammalian cells, DNA methyltransferases (DNMTs) are the enzymes responsible for DNA methylation at the carbon-5 position of cytosine residues situated adjacent to a guanine residue (CpG site), resulting in the formation of 5'-methylcytosine. Our next objective was to assess if the changes in global DNA methylation observed in SNI-rats associated parallel changes in the expression of the two main *de novo* DNMTs (DNMT3a and DNMT3b) and the maintenance DNMT (DNMT1) (Zhao *et al*., 2017) in the DRG and SDH. Compared with sham-rats, the expression levels of DNMT3a significantly increased in the DRG of 10 days SNI-rats (One-way ANOVA: $F_{(2, 10)}$ =5.72, p<0.05; *SNI Day 10 vs. sham* p<0.05) (**Fig. 22A**). DNMT3b levels were significantly higher in the DRGs of 5 days SNI rats than in sham-rats (One-way ANVA: $F_{(4, 20)}$ =10.59 p<0.001: *SNI Day 5 vs. sham* p<0.001; *SNI Day 5 vs. SNI Day 10* p<0.001) (**Fig. 22B**). In the DRGs of 10 days SNI-rats, there was a significant decreased in the expression levels of DNMT3b compared to 5 days SNI-rats (p< 0.001), and the values were similar to those from sham-rats.

In the SDH, no differences were observed in the expression levels of DNMT3a (**Fig. 22D**) but DNMT3b expression was significantly higher in SNI-rats sacrificed 5 and 10 days after the surgery than in sham-rats (One-way ANOVA: $F_{(2, 16)}$ =13.09, p<0.001: *SNI Day 5 vs. sham* p<0.001; *SNI Day 10 vs. sham* p<0.05) (**Fig. 22E**). No differences were observed in the expression levels of DNMT1 in the DRGs and SDH of 5 or 10 days after the nerve injury (**Fig. 22C** and **F**).

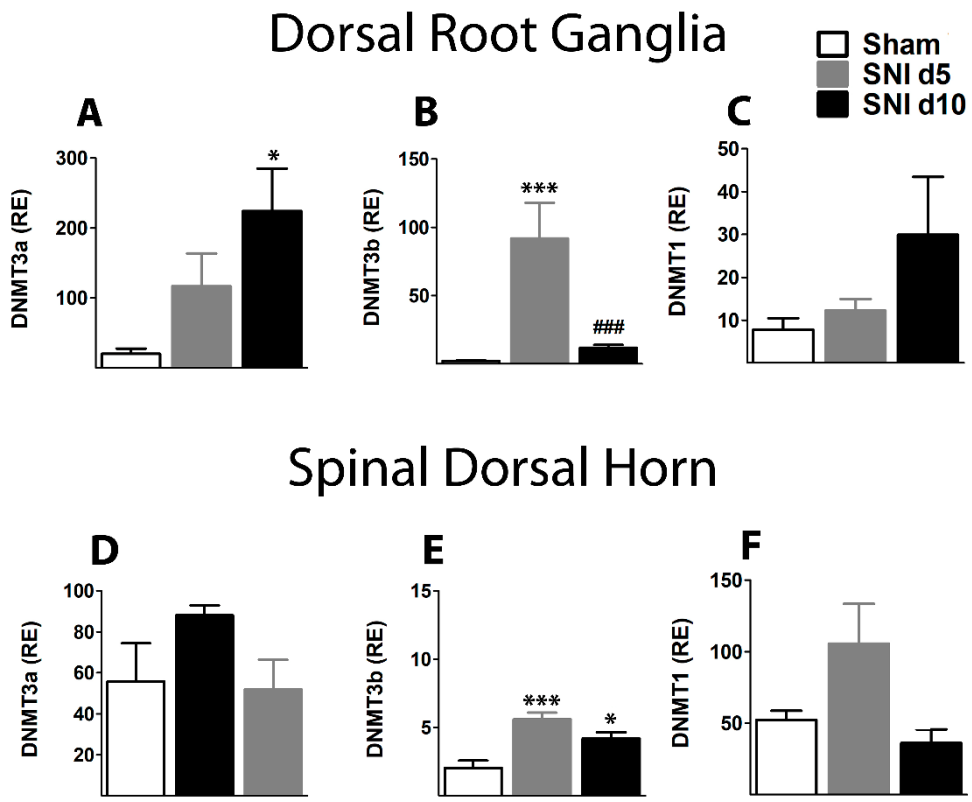


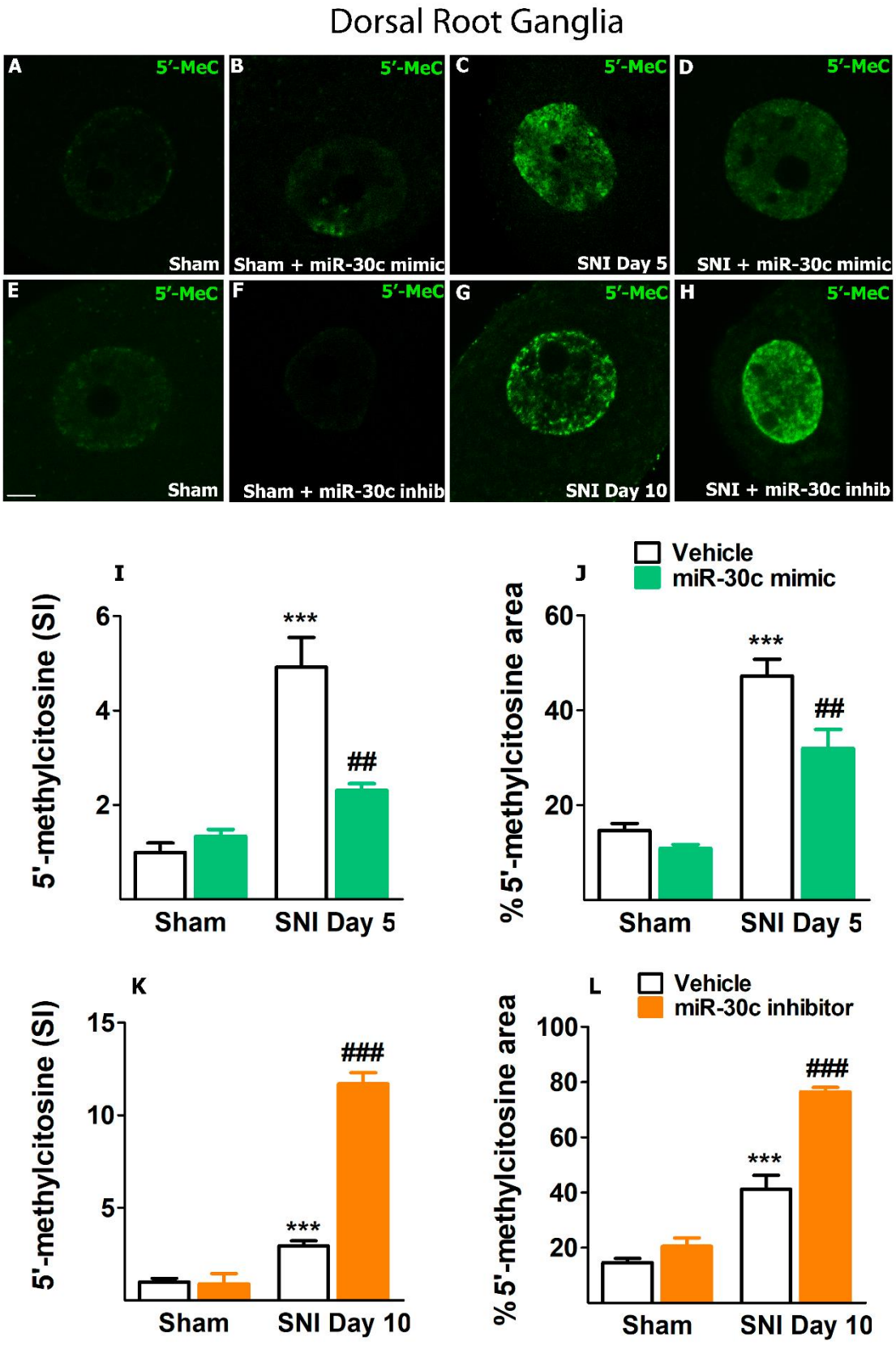
Figure 22. Changes induced by sciatic nerve injury in the mRNA expression levels of DNMT3a, DNMT3b and DNMT1 in the dorsal root ganglia and spinal dorsal horn in rats. mRNA relative expression (vs 18S) of DNMT3a, DNMT3b and DNMT1 in the dorsal root ganglia (DRG) (**A**, **B** and **C**) and in the spinal dorsal horn (SDH) (**D**, **E** and **F**), determined on day 5 and 10 after SNI (n=8) or sham surgery (n=8). *p<0.05, ***p<0.001 vs sham; ###p<0.001 vs SNI Day 5. One-way ANOVA.

1.3 miR-30c-5p mimic and miR-30c-5p inhibitor modulate global DNA methylation in the rat dorsal root ganglion and spinal dorsal horn neurons after sciatic nerve injury in rats

Given the long lasting or even permanent functional consequences of miR-30c-5p modulation in rats subjected to SNI (Tramullas *et al*., 2018 and present results), our next objective was to assess if miR-30c-5p was involved in the aberrant DNA methylation associated with chronic neuropathic pain. For this purpose, we measured global DNA methylation levels (5'methylcitosine immunostaining) in isolated neurons of the DRG and SDH from SNI-rats treated with miR-30c-5p mimic, miR-30c-5p inhibitor or vehicle, following the same protocol of administration described before (**Fig. 19A** and **B**).

5'-MeC immunostaining in the DRG neurons from sham-rats treated with vehicle (**Fig. 23A**) or miR-30c-5p mimic (**Fig. 23B**) was detected in typical heterochromatin areas such as the perinuclear regions whereas 5 days SNI-rats exhibited a homogeneous and increased 5'-MeC immunostaining (**Fig. 23C**). DRG neurons from SNI-rats treated with miR-30c-5p mimic showed small accumulations of 5'-methyltilcitosine on a diffuse background within the nucleoplasm (**Fig. 23D**). The averages of the signal intensity (SI) (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 6)}$ =26.41, p<0.01, "nerve injury": $F_{(1, 6)}$ =72.7, p<0.001; "treatment": $F_{(1, 6)}$ =15.56, p<0.01) and the percentage of methylated area (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 6)}$ =6.49, p<0.05, "nerve injury": $F_{(1, 6)}$ =143, p<0.001; "treatment": $F_{(1, 6)}$ =18.06, p<0.01) were significantly lower than the ones exhibited by SNI-rats sacrificed 5 days after the surgery (**Fig. 23I** and **J**).

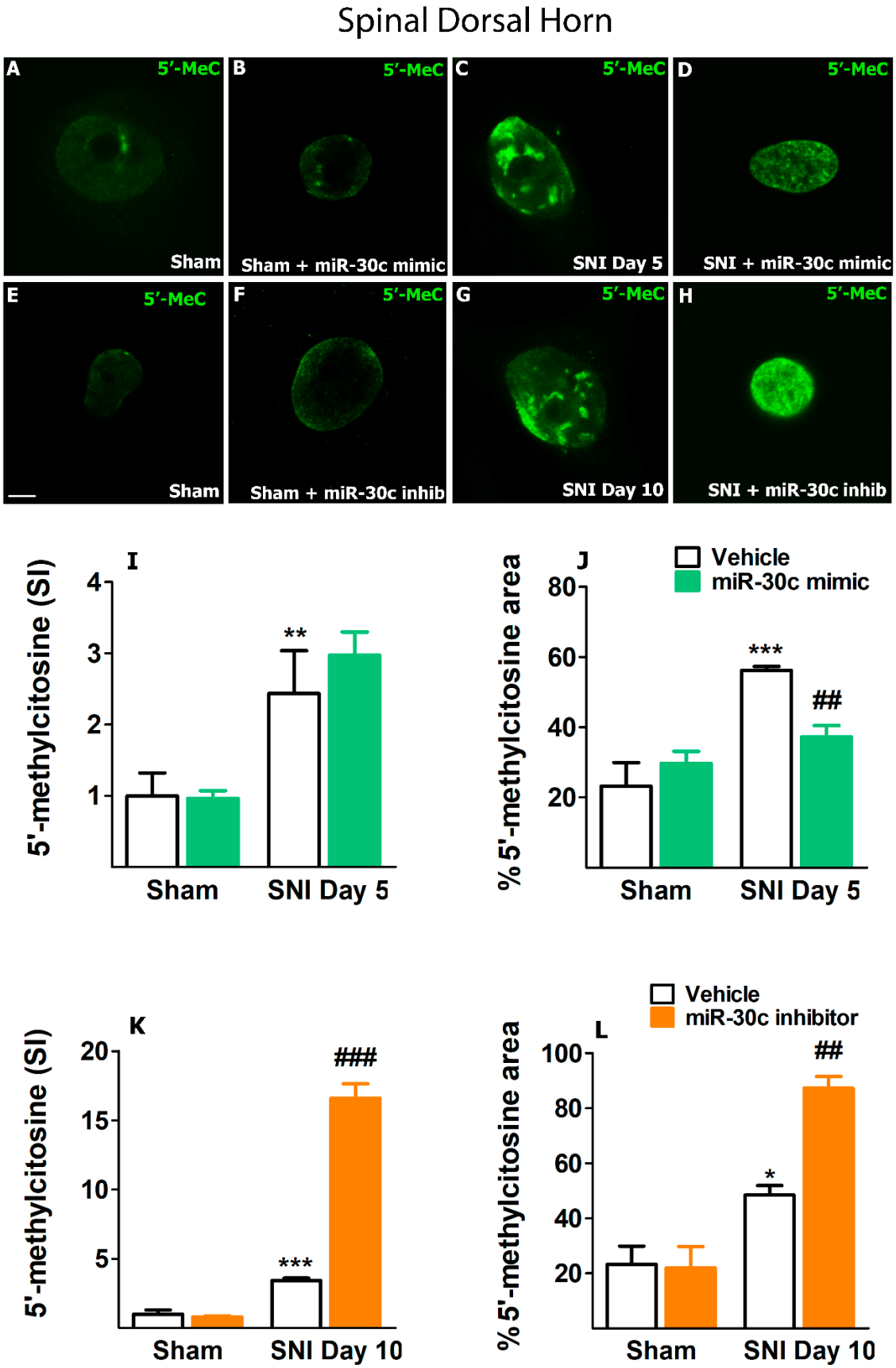
DRG neurons of 10 days SNI-rats exhibited a homogeneous distribution of 5'-MeC that was significantly higher than sham-rats treated with vehicle or miR-30c-5p inhibitor (**Fig. 23E-G).** Further, SNI-rats treated with miR-30c-5p inhibitor showed intense 5'-MeC immmunoreactive signals, homogeneously distributed through all the nucleoplasm except in areas lacking DNA (**Fig. 23H**). The averages of DNA methylated area (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 8)}$ =21.92, p<0.01, "nerve injury": $F_{(1, 8)}$ =172, p<0.001; "treatment": $F_{(1, 8)}$ =42.59, p<0.001) and fluorescence intensity (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)}$ =106.4, p<0.001, "nerve injury": $F_{(1, 7)}$ =219.7, p<0.001; "treatment": $F_{(1, 7)}$ =101.0, p<0.001) were significantly higher in the neurons from SNI-rats treated with miR-30c-5p inhibitor than those of 10 days SNI-rats treated with vehicle (**Fig. 23K** and **L).**


Dorsal Root Ganglia
A 5′-MeC Sham
B 5′-MeC Sham + miR-30c mimic
C 5′-MeC SNI Day 5
D 5′-MeC SNI + miR-30c mimic
E 5′-MeC Sham
F 5′-MeC Sham + miR-30c inhib
G 5′-MeC SNI Day 10
H 5′-MeC SNI + miR-30c inhib
I
5'-methylcitosine (SI)
***
##
Sham
SNI Day 5
J
Vehicle
miR-30c mimic
% 5'-methylcitosine area
***
##
Sham
SNI Day 5
K
5'-methylcitosine (SI)
***
###
Sham
SNI Day 10
L
Vehicle
miR-30c inhibitor
% 5'-methylcitosine area
***
###
Sham
SNI Day 10

SDH sensory neurons from sham-rats treated with vehicle (**Fig. 24A**) or miR-30c-5p mimic (**Fig. 24B**) exhibited low immunoreactivity 5'-MeC whereas 5 days SNI-rats showed dense accumulations of 5'-MeC though the nucleoplasm (**Fig. 24C**). SNI-rats treated with miR-30c-5p mimic (**Fig. 24D)** showed a homogenous distribution of 5'methylcitosine immunostaining whose signal intensity was similar to the observed in the 5 days SNI-rats treated with vehicle and sacrificed on day 5 (Two-way ANOVA: "nerve injury": $F_{(1,7)} = 17.83$, $p<0.01$). The percentage of methylated area was significantly lower in SNI-rats treated with miR-30c-5p mimic when compared to SNI-rats treated with vehicle (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)} =13.25$, $p<0.01$, "nerve injury": $F_{(1, 7)} =33.59$, $p<0.001$) (**Fig. 24I** and **J**).

SDH sensory neurons from 10 days SNI-animals also showed dense accumulations of 5'-MeC distributed near the nucleolar envelope and the nucleoplasm that were higher than SDH neurons from sham-rats treated with either vehicle or miR-30c-5p inhibitor (**Fig. 24E-G**). In addition, 5'-methylcitosine immunoreactivity (signal intensity and percentage of stained area) was also higher in the SDH sensory neurons from SNI-rats treated with miR-30c-5p inhibitor (**Fig. 24H**) than in the neurons isolated from SNI-rats treated with vehicle and sacrificed on day 10 [*Signal intensity*: (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)} =124.8$, $p<0.001$, "nerve injury": $F_{(1, 7)} =231.8$, $p<0.001$; "treatment": $F_{(1, 7)} =117.7$, $p<0.001$); *5'-MeC methylated area*: (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)} =12.03$, $p<0.05$, "nerve injury": $F_{(1, 7)} =61.07$, $p<0.001$; "treatment": $F_{(1, 7)} =10.56$, $p<0.05$)] (**Fig. 24K** and **L**).

Figure 23. Effects of the treatment with miR-30c-5p mimic or miR-30c-5p inhibitor on the global DNA methylation in dorsal root ganglion neurons from rats subjected to sciatic nerve injury. Representative images showing 5'-methylcitosine (5´-MeC)-positive immunostaining in neurons isolated from the dorsal root ganglia (DRG) of sham-rats treated with vehicle (**A**) or miR-30c-5p mimic (**B**), SNI-rats sacrificed on day 5 treated with vehicle (**C**) or miR-30c-5p mimic (**D**), Sham-rats treated with vehicle (**E**) or miR-30c-5p inhibitor (**F**) and SNI-rats sacrificed on day 10 treated with vehicle (**G**) or miR-30c-5p inhibitor (**H**). No positive signals were detected in DNA lacking areas such as the speckles. The average intensity of the signal (SI) **(I** and **J)** and the percentage of methylated area **(K** and **L)** were determined in 60 neurons (20 neurons per rat, 3 rats per group). *** $p<0.001$ vs Sham; ## $p<0.01$, ### $p<0.001$ vs SNI (Two-way ANOVA followed by Bonferroni post hoc test). Scale bar: 5 μm


Spinal Dorsal Horn
A
5′-MeC
Sham
B
5′-MeC
Sham + miR-30c mimic
C
5′-MeC
SNI Day 5
D
5′-MeC
SNI + miR-30c mimic
E
5′-MeC
Sham
F
5′-MeC
Sham + miR-30c inhib
G
5′-MeC
SNI Day 10
H
5′-MeC
SNI + miR-30c inhib
I
5'-methylcitosine (SI)
4
3
2
1
**
Sham
SNI Day 5
J
Vehicle
miR-30c mimic
% 5'-methylcitosine area
80
60
40
20
***
##
Sham
SNI Day 5
K
5'-methylcitosine (SI)
20
15
10
5
###
***
Sham
SNI Day 10
L
Vehicle
miR-30c inhibitor
% 5'-methylcitosine area
100
80
60
40
20
##
*
Sham
SNI Day 10

These results indicate that the long lasting antiallodynic effect induced by miR-30c-5p inhibitor in SNI-rats is associated with a strong global hyper-methylation of the DNA in both DRG nociceptors and SDH sensitive neurons. On the other hand, the hyperalgesic state induced by the treatment of SNI-rats with miR-30c-5p mimic did not associated quantitative changes in the global DNA methylation of nociception-related neurons versus SNI-rats treated with vehicle.

1.3.1 miR-30c-5p mimic and miR-30c-5p inhibitor modulate the expression of DNMT3a, DNMT3b and DNMT1 in dorsal root ganglion and spinal dorsal horn neurons after sciatic nerve injury in rats

Online software resources based on bioinformatic algorithms, including TargetScan 4.0 and miRanda, predict DNMT3b as a possible target for miR-30c-5p. Therefore, our next objective was to assess if there was a relationship between miR-30c-5p modulation and changes in the expression levels of DNMTs after SNI. Therefore, we evaluated the changes in the mRNA expression levels of the predicted target DNMT3b, as well as DNMT3a and DNMT1 in the DRG and SDH, in SNI-rats treated with miR-30c-5p inhibitor, miR-30c-5p mimic or vehicle. Results from **chapter 1.3** were included here to facilitate the understanding of the global results by the reader.

Figure 24. Effects of the treatment with miR-30c-5p mimic or miR-30c-5p inhibitor on the global DNA methylation in the spinal dorsal horn neurons from rats subjected to sciatic nerve injury. Representative images showing 5'-methylcitosine (5´-MeC)-positive immunostaining in neurons isolated from the spinal dorsal horn (SDH) of sham-rats treated with vehicle (**A**) or miR-30c-5p mimic (**B**), SNI-rats sacrificed on day 5 treated with vehicle (**C**) or miR-30c-5p mimic (**D**), Sham-rats treated with vehicle (**E**) or miR-30c-5p inhibitor (**F**) and SNI-rats sacrificed on day 10 treated with vehicle (**G**) or miR-30c-5p inhibitor (**H**).No positive signals were detected in DNA lacking areas such as the speckles. The average intensity of the signal (SI) **(I** and **J)** and the percentage of methylated area **(K** and **L)** were determined in 60 neurons (20 neurons per rat, 3 rats per group). *p<0.05, **p<0.01, *** p<0.001 vs Sham; ## p<0.01, ### p<0.001 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test). Scale bar: 5 μm.

As shown in **Figure 25 A-C**, SNI-rats that received miR-30c-5p mimic showed a significant reduction in DNMT3a (Two-way ANOVA: "nerve injury": $F_{(1,10)}$ =5.69, p<0.05; "treatment": $F_{(1, 10)}$ =6.25, p<0.05) and DNMT3b (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 9)}$ =5.23, p<0.05; "nerve injury": $F_{(1, 9)}$ =7.95 p<0.05; "treatment": $F_{(1, 9)}$ =5.30, p<0.05) expression levels in the DRG compared to SNI-rats treated with vehicle and sacrificed on day 5 after the surgery. No significant changes were observed in DNMT1 expression levels, and treatment with miR-30c-5p mimic in sham-rats did not alter the levels of any of the enzymes.

In contrast, as shown in **Figure 25 D-F**, the treatment of SNI-rats with miR-30c-5p inhibitor led to a significant increase in the expression levels of all the DNMTs analyzed in the DRG [Two-way ANOVA: *(DNMT3a*: "treatment x nerve injury": $F_{(1, 11)}$ =10.12, p<0.01; "nerve injury": $F_{(1, 11)}$ =18.77 p<0.01; "treatment": $F_{(1, 11)}$ =9.88, p<0.01), (*DNMT3b*: "nerve injury": $F_{(1, 14)}$ =22.10, p<0.001) and (*DNMT1*: "nerve injury": $F_{(1, 12)}$ =9.21, p<0.05) when compared to SNI rats treated with vehicle. In sham-rats, the treatment with miR-30c-5p inhibitor did not alter the levels of any of the enzymes.

Protein expression of DNMT3a and DNMT3b (**Figure 26**) analyzed by western-blot in DRG lysates of the same group of animals revealed a similar pattern expression to the one described for the gene expression, where SNI-rats treated with miR-30c-5p inhibitor exhibited an increase in the protein levels of both DNMT3a and DNMT3b (DNMT3a: SNI Day 10: 0.98± 0.01; SNI + miR-30c-5p inhibitor: 6.35±1.39; t=5.42, p<0.05, SNI day 10 vs SNI + miR-30c-5p inhibitor) (DNMT3b: t=4.22, p<0.05, SNI day 10 vs SNI + miR-30c-5p inhibitor).

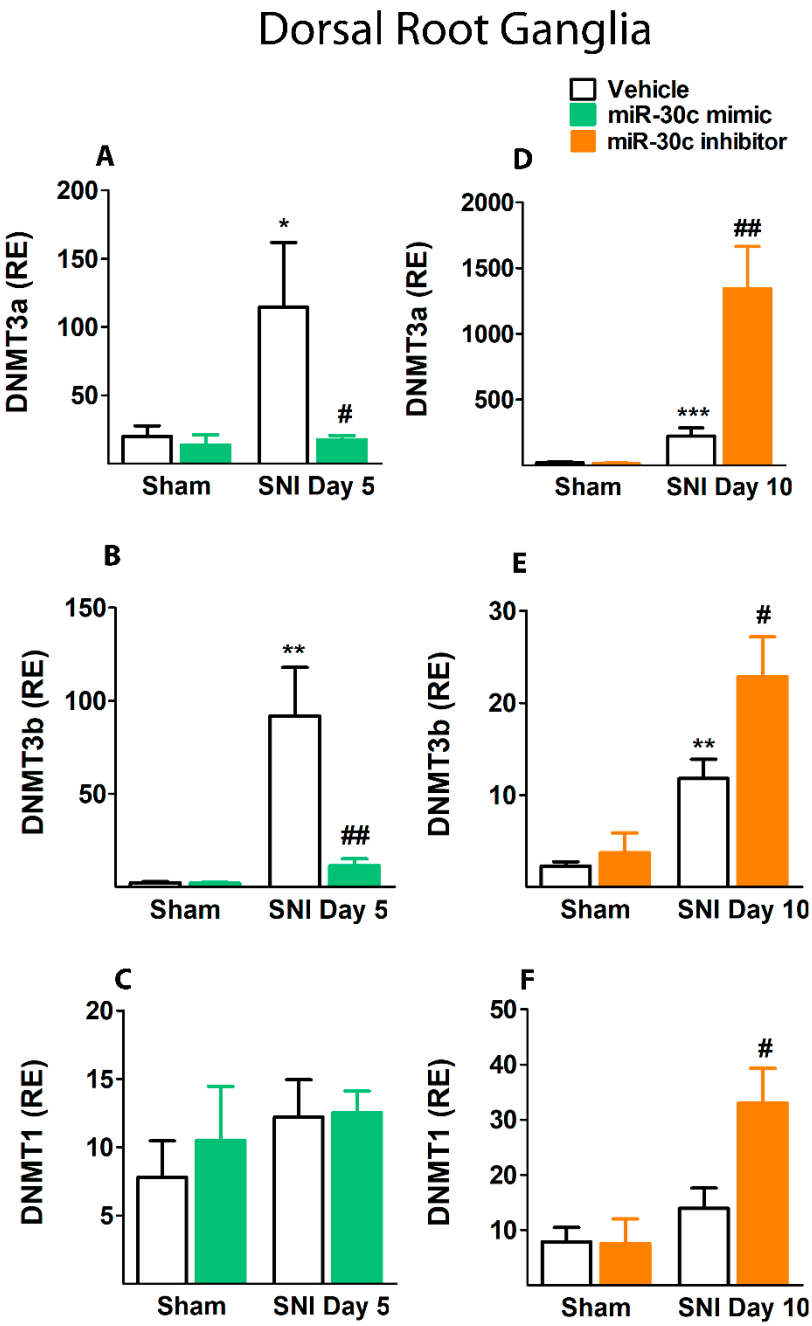


Figure 25. Effects of the treatment with miR-30c-5p mimic or miR-30c-inhibitor 5p on the expression of DNMT3a, DNMT3b and DNMT1 in dorsal root ganglion from rats subjected to sciatic nerve injury. mRNA relative expression (vs 18S) of DNMT3a (**A,D**), DNMT3b (**B, E**) and DNMT1 (**C,F**) in the dorsal root ganglia (DRG) of sham-rats, sham-rats treated with miR-30c-5p mimic or miR-30c-5p inhibitor, SNI-rats sacrificed on day 5 or day 10 treated with vehicle, SNI-rats treated with miR-30c-5p mimic, and SNI-rats treated with miR-30c-5p inhibitor (Sham: n=6; Sham + miR-30c-5p mimic: n=5; Sham + miR-30c-5p inhibitor: n=5; SNI Day 5: n=8; SNI Day 10: n=8; SNI + miR-30c-5p inhibitor: n=6; SNI + miR-30c-5p mimic: n=6).*p<0.05, **p<0.01, ***p<0.001 vs Sham; # p<0.05, ## p<0.001 vs SNI, (Two-way ANOVA followed by Bonferroni post hoc test

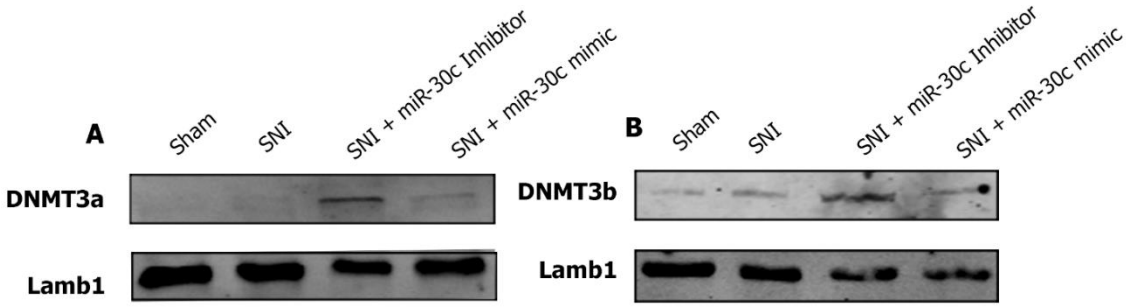


Figure 26. Protein expression of DNMT3a and DNMT3b in the DRG determined by western blot (WB) analysis in sham-rats, SNI-rats, SNI-rats with miR-30c-5p inhibitor and SNI-rats with miR-30c-5p mimic. SNI-rats treated with miR-30c-5p inhibitor exhibited an increase in the protein levels of DNMT3a (**A**) and DNMT3b (**B**).

Figure 27A-C shows the expression levels of DNMT3a, DNMT3b and DNMT1 in the SDH of SNI-rats that received miR-30c-5p mimic. No differences were observed in the expression of any of the enzymes after the treatment with miR-30c-5p mimic when compared to SNI-rats treated with vehicle and sacrificed 5 days after the nerve injury. Thus, the overexpression of these DNMTs induced by SNI was not affected by the treatment with miR-30c-5p-mimic in the SDH. In contrast, SNI-rats treated with miR-30c-5p inhibitor significantly increased the mRNA expression of DNMT3b (Two-way ANOVA: "nerve injury": $F_{(1,\ 12)}$ =8.65, $p<0.05$) and DNMT1 (Two-way ANOVA: "treatment x nerve injury": $F_{(1,\ 17)}$ =12.75, $p<0.01$; "treatment": $F_{(1,\ 17)}$ =7.08, $p<0.05$) when compared to those SNI-rats treated with vehicle in the SDH. No significant changes we observed in DNMT3a relative expression and in sham rats, treatment with miR-30c-5p inhibitor did not alter the levels of any of the enzymes (**Fig. 27D-F**).

Protein expression of DNMT3a and DNMT3b (**Figure 28**) analyzed by western-blot in SDH lysates of the same group of animals revealed a similar pattern expression to the one described for the gene expression, while no changes were observed in DNMT3a protein levels, SNI-rats with miR-30c-5p inhibitor exhibited an increase in the protein levels of DNMT3b (DNMT3b: SNI Day 10: 0.98± 0.01; SNI + miR-30c-5p inhibitor: 4.82±0.81) (SNI day 10 vs SNI + miR-30c-5p inhibitor ($t=6.67$, $p<0.05$).

Increased mRNA expression after the treatment with miR-30c-5p inhibitor is the expected regulation if such mRNAs were miR-30c-5p targets. These

results also agree with the increase in the global DNA methylation featured by this by this group of rats (**Fig. 23** and **24**).

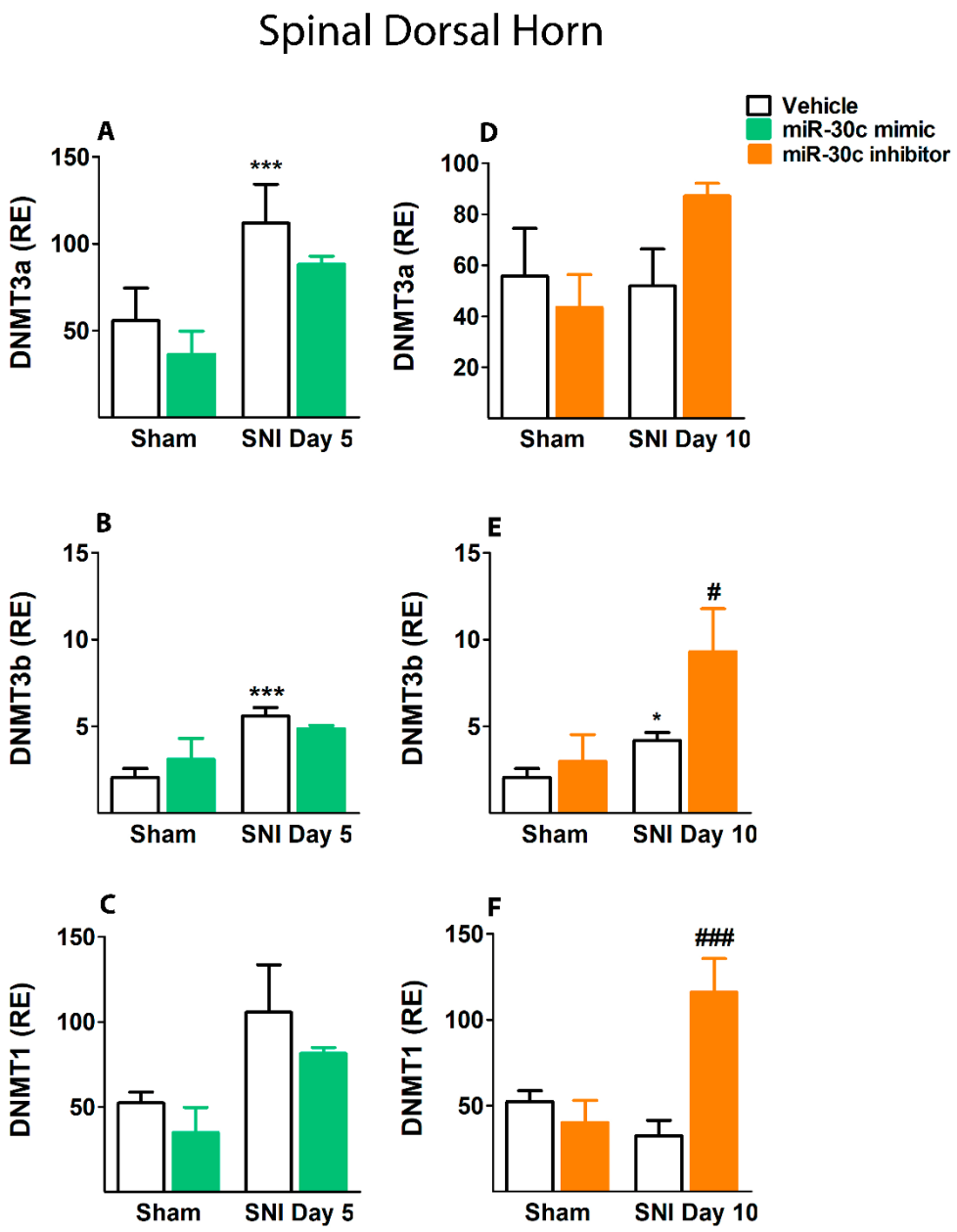


Figure 27. Effects of the treatment with miR-30c-5p mimic or miR-30c-5p inhibitor on the expression of DNMT3a, DNMT3b and DNMT1 in the spinal dorsal horn from rats subjected to sciatic nerve injury. mRNA relative expression (vs 18S) of DNMT3a (**A,D**), DNMT3b (**B, E**) and DNMT1 (**C,F**) in the spinal dorsal horn (SDH) of sham-rats, sham-rats treated with miR-30c-5p mimic or miR-30c-5p inhibitor, SNI-rats sacrificed on day 5 or day 10 treated with vehicle, SNI-rats treated with miR-30c-5p mimic, and SNI-rats treated with miR-30c-5p inhibitor (Sham: n=6; Sham + miR-30c-5p mimic: n=5;Sham + miR-30c-5p inhibitor: n=5; SNI Day 5: n=8; SNI Day 10: n=8; SNI + miR-30c-5p inhibitor: n=6; SNI + miR-30c-5p mimic: n=6). ***p<0.001 vs Sham; # p<0.05, ### p<0.001 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test).

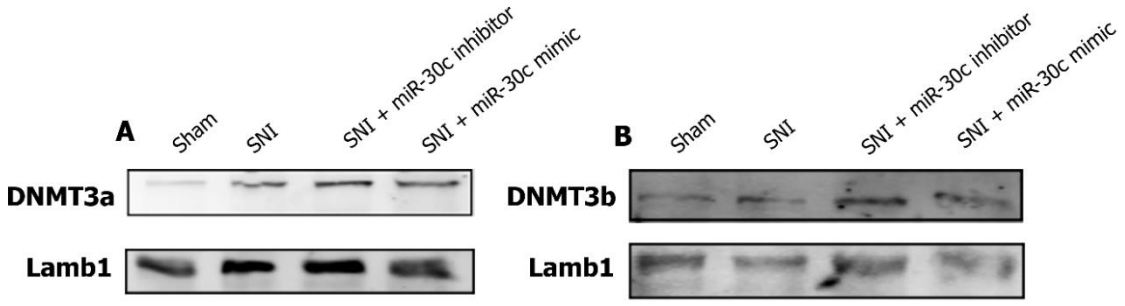


Figure 28. Protein expression of DNMT3a and DNMT3b in the SDH determined by western blot (WB) analysis in Sham-rats, SNI-rats, SNI-rats with miR-30c-5p inhibitor and SNI-rats with miR-30c-5p mimic. SNI-rats treated with miR-30c-5p inhibitor exhibited an increase in the protein levels of DNMT3a (**A**) and DNMT3b (**B**)

1.4 Luciferase reporter assays revealed post-transcriptional regulation of DNMT3a and DNMT3b by miR-30c-5p in Hela and SH-SY5Y cells

We further assessed whether DNMT3a and DNMT3b are post-transcriptionally regulated by miR-30c-5p, as predicted by bioinformatic tools and suggested by our data. To this end, pLightSwitch™ or pMIR-REPORT™ luciferase miRNA expression reporter vectors, including the predicted miR-30c-5p binding site, of either the 3'UTR of DNMT3a or the 3'UTR of DNMT3b, respectively, were transiently transfected to human HeLa and SH-SY5Y cell lines. Cells were co-transfected with miR-30c-5p mimic or a scrambled miR-mimic, in parallel experiments (three independent assays with triplicate measurements). Luciferase activity was assessed 24h thereafter. Our results indicate that miR-30c-5p produced a significant decrease of the luciferase activity in HeLa cell line after the transfection with 25 ng of either DNMT3a-3'UTR ($t=17.57$, $p<0.05$) (**Fig. 29A**) or DNMT3b-3'UTR ($t=18.23$, $p<0.001$) (**Fig. 29B**), compared with those cells transfected with the empty vector, pLightSwitch™ or pMIR-REPORT™, respectively. While miR-30c-5p also produced a significant decrease of the luciferase activity in SH-SY5Y ($t=11.81$, $p<0.001$) (**Fig. 29C**), in our experimental conditions, transfection with DNMT3a-3'UTR was not successful. The scrambled mimic did not modify the luciferase activity. These results suggest that both DNMT3a and DNMT3b are direct mRNA targets of miR-30c-5p in the HeLa and SH-SY5Y human cell line.

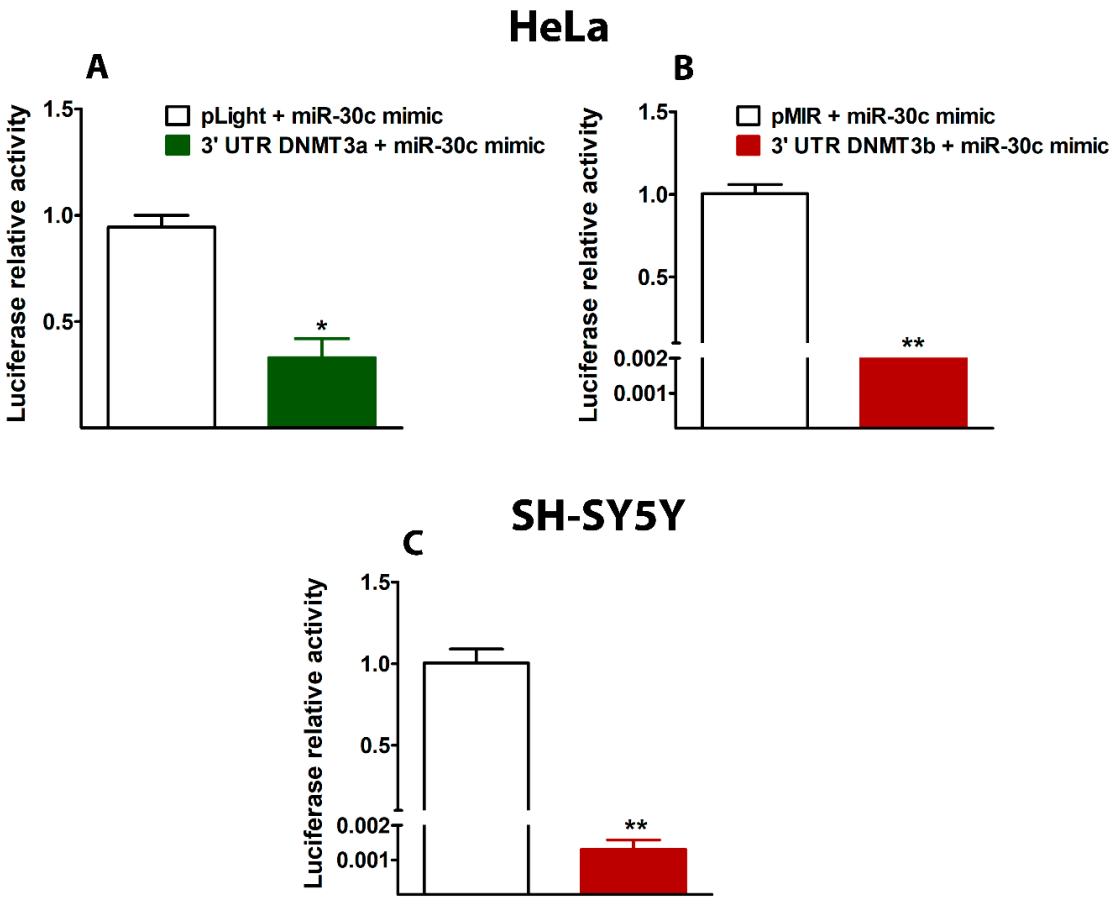


Figure 29. Luciferase reporter assays in HeLa and SH-SY5Y cells co-transfected with pLight-REPORT luciferase vector (25 ng) containing the 3'-UTR of DNMT3a (**A**) and pMIR-REPORT luciferase vector containing the 3'-UTR of DNMT3b (**B** and **C**) and miR-30c-5p mimic (10 nM). The data represent the relative luciferase units normalized to the amount of protein in three independent experiments with triplicate measurements. *p<0.05; **p<0.01, two-tailed Student's t-test.

1.5 Modulation of miR-30c-5p in rats subjected to sciatic nerve injury results in methylation changes of Nfyc and TGFβ-1 in the SDH

The antiallodynic effect of miR-30c-5p inhibitor administered to SNI-rats is very long-lasting (Tramullas *et al*. 2018) and is associated with both down-regulation of miR-30c-5p and DNA hyper-methylation (present results). Therefore, we postulated that the gene coding miR-30c-5p might be subjected to differential epigenetic transcriptional regulation by DNMT-mediated mechanisms in SNI-rats treated with saline, miR-30c-5p inhibitor or miR-30c-5p mimic.

miR-30c-5p is an intragenic intronic miRNA embedded within the Nfyc-coding host gene and transcribed under control of the same promoter. Intronic miRNAs are usually expressed in coordination with the host gene mRNA (Baskerville *et al*.,2005; Kim *et al*.,2007; França *et al*., 2016; Boivin *et al*.,2017). Therefore, our first objective was to demonstrate a coordinated transcriptional regulation of the intronic miR-30c-5p and its host gene Nfyc. Thus, linear regression and correlation analyses indicate that Nfyc mRNA levels correlated directly with those of miR-30c-5p in the SDH (**Fig. 30A**) and DRG (**Fig. 30D**). Moreover, Nfyc and miR-30c-5p expressions featured parallel changes under the different experimental conditions of our study in both SDH [Nfyc: (One-way ANOVA: $F_{(3, 17)}$ =5.04, p<0.05; *Sham vs SNI,* p<0.05; *SNI vs. SNI + miR-30c inhibitor* p<0.05). miR-30c-5p: (One-way ANOVA: $F_{(3, 17)}$ =7.47, p<0.01; *Sham vs SNI,* p<0.05; Sham vs SNI + miR-30c mimic, p<0.05; *SNI vs. SNI + miR-30c inhibitor,* p<0.05)] **Fig. 30B** and **C**) and DRG [Nfyc: (One-way ANOVA: $F_{(3, 19)}$ =12.34, p<0.001; *Sham vs SNI,* p<0.01; *Sham vs SNI + miR-30c mimic, p<0.01; SNI vs SNI + miR-30c inhibitor p<0.01*). miR-30c-5p: (One-way ANOVA: $F_{(3, 17)}$ =7.04, p<0.01; *Sham vs SNI,* p<0.05; *SNI vs. SNI + miR-30c inhibitor,* p<0.05; Sham vs SNI + miR-30c mimic, p<0.05)] ((**Fig. 30E** and **F**).

Once the parallel expression of Nfyc and miR-30c-5p was confirmed, we proceeded to assess DNA methylation changes in the promoter region of the gene coding Nfyc induced by miR-30c-5p modulation in the SDH and DRG from SNI-rats. We used the methylation sensitive qPCR (ms-qPCR), a quantitative DNA methylation assay that allows to evaluate the percentage of methylation of the promoter of a given gene (Oakes *et al*., 2016). In our experimental condition, we were unable to perform these experiments in DRGs due to the amount of sample needed for the genomic DNA extraction. Quantitative analysis (**Fig. 31A**) reveal that, compared with vehicle, the treatment of SNI-rats with miR-30c-5p inhibitor produced a strong increase in the percentage of methylation of the promoter of Nfyc in the SDH (One-way ANOVA: $F_{(3, 18)}$ =5.09, p<0.05; *SNI vs. SNI + miR-30c inhibitor* p<0.05). These results support an epigenetic autoregulation of miR-30c-5p transcription Therefore, hyper-methylation of the promoter would result in a transcriptional repression of Nfyc and, consequently, in the negative

regulation of miR-30c-5p and Nfyc expressions, as it occurs in SNI-rats treated with the miRNA inhibitor. (**Fig. 29B** and **C**).

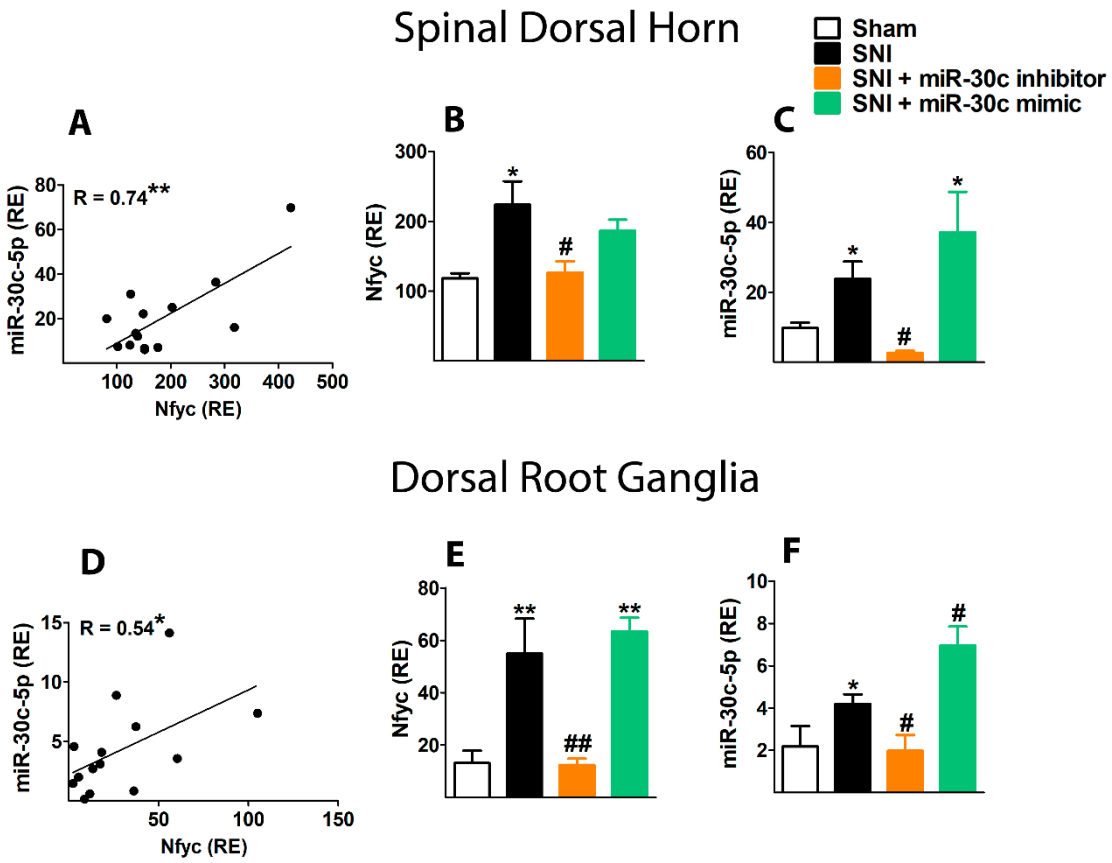


Figure 30. Effects of the treatment with miR-30c-5p inhibitor or miR-30c-5p mimic on the expression of miR-30c-5p and its host gene Nfyc in the spinal dorsal horn and dorsal root ganglia from rats subjected to sciatic nerve injury. A and **D:** Linear regression and Pearson's correlation analyses showing the relationship of miR-30c-5p relative expression with Nfyc mRNA levels in the spinal dorsal horn (SDH) and dorsal root ganglia (DRG). R: Pearson's correlation coefficient. Nfyc mRNA (**B** and **E**) and miR-30c-5p (**C** and **F**) expression in sham and SNI-rats treated with vehicle, miR-30c-5p inhibitor or miR-30c-5p mimic. (n = 5 rats per group) *p<0.05, **p<0.01 vs sham; # p<0.05, ## p<0.01 vs SNI. One-way ANOVA followed by Bonferroni post hoc test).

Previous results of our group show that the anti-inflammatory cytokine TGFβ-1 constitutes a direct transcriptional target of miR-30c-5p, which mediates the antiallodynic effect of miR-30c-5p inhibitor in SNI-rats (Tramullas *et al.*, 2018). Therefore, we considered of great interest to explore the effects of miR-30c-5p modulation on the epigenetic control of TGFβ-1 transcription in SNI-rats. As shown in **Figure 31B**, the percentage of methylation of the promoter of TGFβ-1 increased significantly in neuropathic SNI-rats treated with vehicle (One-way ANOVA: $F_{(3,\ 15)} = 13.10$, $p<0.001$; *Sham vs SNI,*

p<0.001; SNI vs. SNI + miR-30c mimic, p<0.001; SNI vs SNI + miR-30c inhibitor p<0.001), in comparison with sham rats. In contrast, pain-free SNI-rats treated with miR-30c-5p inhibitor showed promoter hypo-methylation when compared with neuropathic SNI-rats. Therefore, our results support that the methylation state of the TGFβ-1 promoter is regulated by miR-30c-5p related mechanisms with long-term consequences on pain perception.

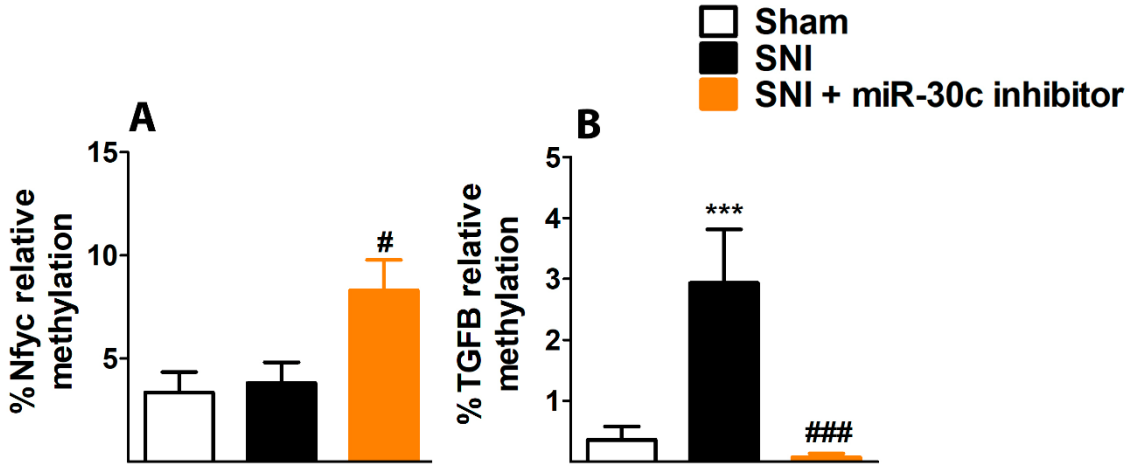


Figure 31. Effects of the treatment with miR-30c-5p inhibitor or miR-30c-5p mimic on the methylation of the promoters of Nfyc and TGF-β1 in the spinal dorsal horn from rats subjected to sciatic nerve injury. Percentage of methylation of the promoters of genes coding Nfyc (**A**) and TGF-β1 (**B**) in the spinal dorsal horn (SDH) from SNI-rats treated with vehicle, miR-30c-5p inhibitor or miR-30c-5p mimic. (n = 5 rats per group) ***p<0.001 vs. Sham; #p<0.05, ###p<0.001 vs SNI. One-way ANOVA followed by Bonferroni post hoc test.

1.6 Trimethylation of lysine 9 on histone H3 (H3K9me3) in dorsal root ganglion and spinal dorsal horn neurons after sciatic nerve injury in rats and its modulation by miR-30c-5p mimic and miR-30c-5p inhibitor.

H3K9 is a common target of methylation *in vivo*; it can carry one, two or three methyl groups. Di- and tri-methylation of H3K9 (H3K9me2/H3K9me3) are epigenetic marks of silent genes and heterochromatin (the condensed, transcriptionally inactive state of chromatin).

Our next objective was to evaluate if neuropathic pain induces changes in the trimethylation levels of H3K9me3 and its modulation by miR-30c-5p. The distribution pattern of H3K9me3 was determined in isolated neurons of the DRG and SDH from sham-rats treated with vehicle, miR-30c-5p mimic or miR-

30c-5p inhibitor and SNI-rats treated with vehicle, miR-30c-5p inhibitor or miR-30c-5p mimic. Neurons were labelled by immunofluorescence with an antibody anti-H3K9me3.

As shown in **Fig. 32A** and **B**, DRG neurons from sham-rats treated with vehicle and miR-30c-5p mimic showed low H3K9me3 immunoreactivity signals and methylated areas that significantly increased in SNI-rats sacrificed 5 days after the surgery (**Fig. 32C**). However, signal intensity (One-way ANOVA: $F_{(2, 8)} = 70.0$, $p<0.001$: *SNI Day 5 vs. SNI Day 10* $p<0.001$) and H3K9me3 methylated area (One-way ANOVA: $F_{(2, 8)} = 45.80$, $p<0.001$; *SNI Day 5 vs. SNI Day 10* $p<0.001$) decreased 10 days after the surgery when compared to 5 days SNI-animals. In addition, SNI-rats treated with miR-30c-5p mimic (**Fig. 32D**) showed a significant reduction in both signal intensity (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 6)} = 45.1$, $p<0.001$; "nerve injury"; $F_{(1, 6)} = 38.66$, $p<0.001$; "treatment": $F_{(1, 6)} = 45.67$, $p<0.001$) and methylated area of H3K9me3 (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 6)} = 22.4$, $p<0.01$; "nerve injury": $F_{(1,6)} = 28.82$, $p<0.01$; "treatment": $F_{(1, 6)} = 17.34$, $p<0.01$) when compared to SNI-rats sacrificed on Day 5 after the nerve injury. No significant changes where observed in Sham-rats treated with miR-30c-5p mimic (**Fig. 32I** and **J**).

SNI-rats treated with vehicle and sacrificed 10 days after the nerve injury did not exhibit significant changes in the signal intensity or H3K9me3 methylated area when compared to sham-rats treated with vehicle. The treatment with miR-30c-5p inhibitor did not modify the H3K9me3 immunoreactivity in sham-rats. (**Fig. 32G-F**). Interestingly, SNI-rats treated with miR-30c-5p inhibitor exhibited a strong increase in the H3K9me3-immunoreactive signal and dense accumulations through all the nucleoplasm (**Fig. 32G**). The intensity of the fluorescent signal (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)} = 28.91$, $p<0.01$, "nerve injury": $F_{(1, 7)} = 37.54$, $p<0.001$; "treatment": $F_{(1, 7)} = 29.42$, $p<0.001$) and the H3K9me3 methylated area (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)} = 217.1$, $p<0.001$, "nerve injury": $F_{(1, 7)} = 250.2$, $p<0.001$; "treatment": $F_{(1, 7)} = 230.7$, $p<0.001$) were significantly higher after the treatment with miR-30c-5p inhibitor compared to SNI-rats treated with vehicle and sacrificed 10 days after the surgery (**Fig. 32K** and **L**).

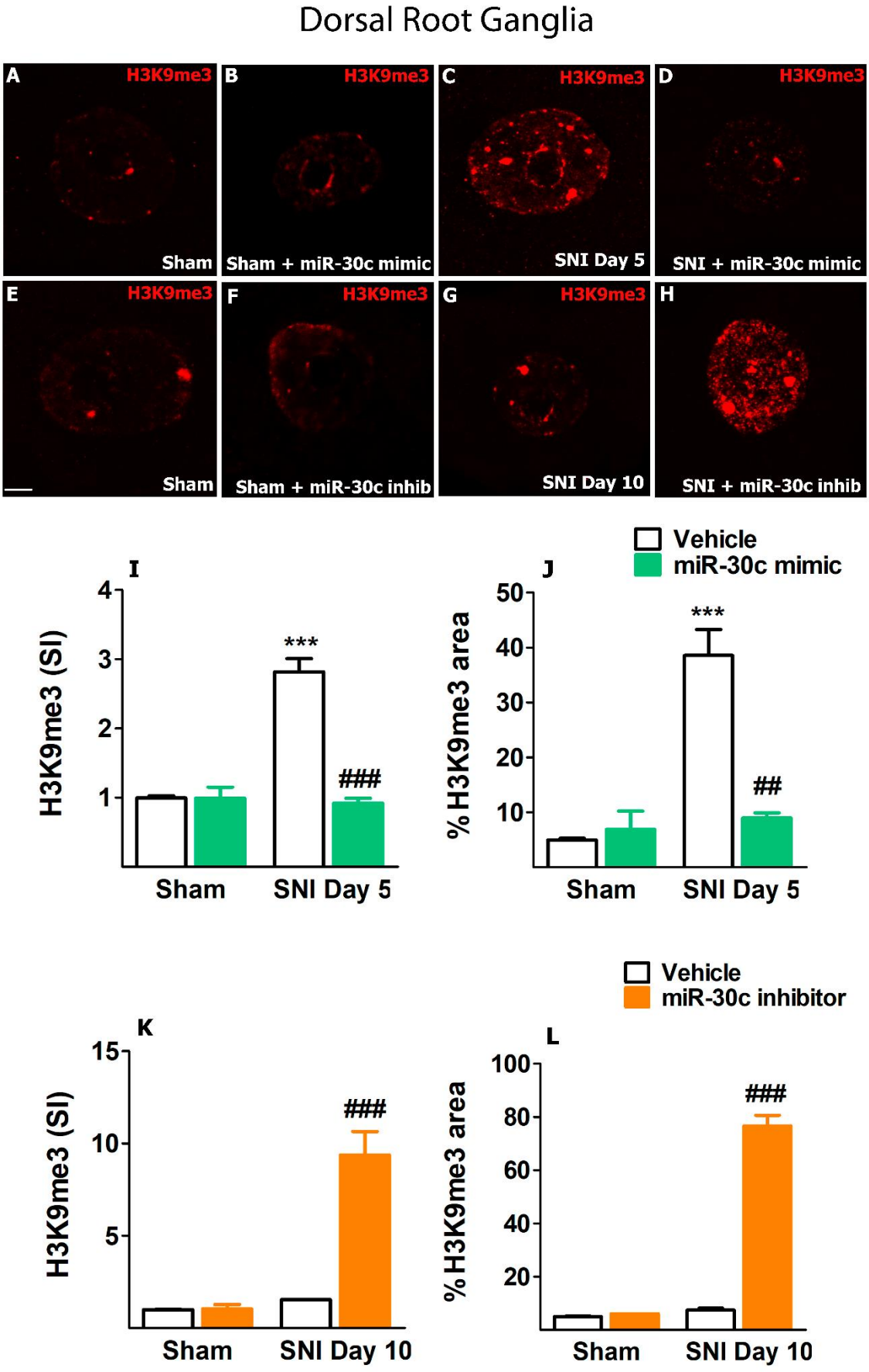
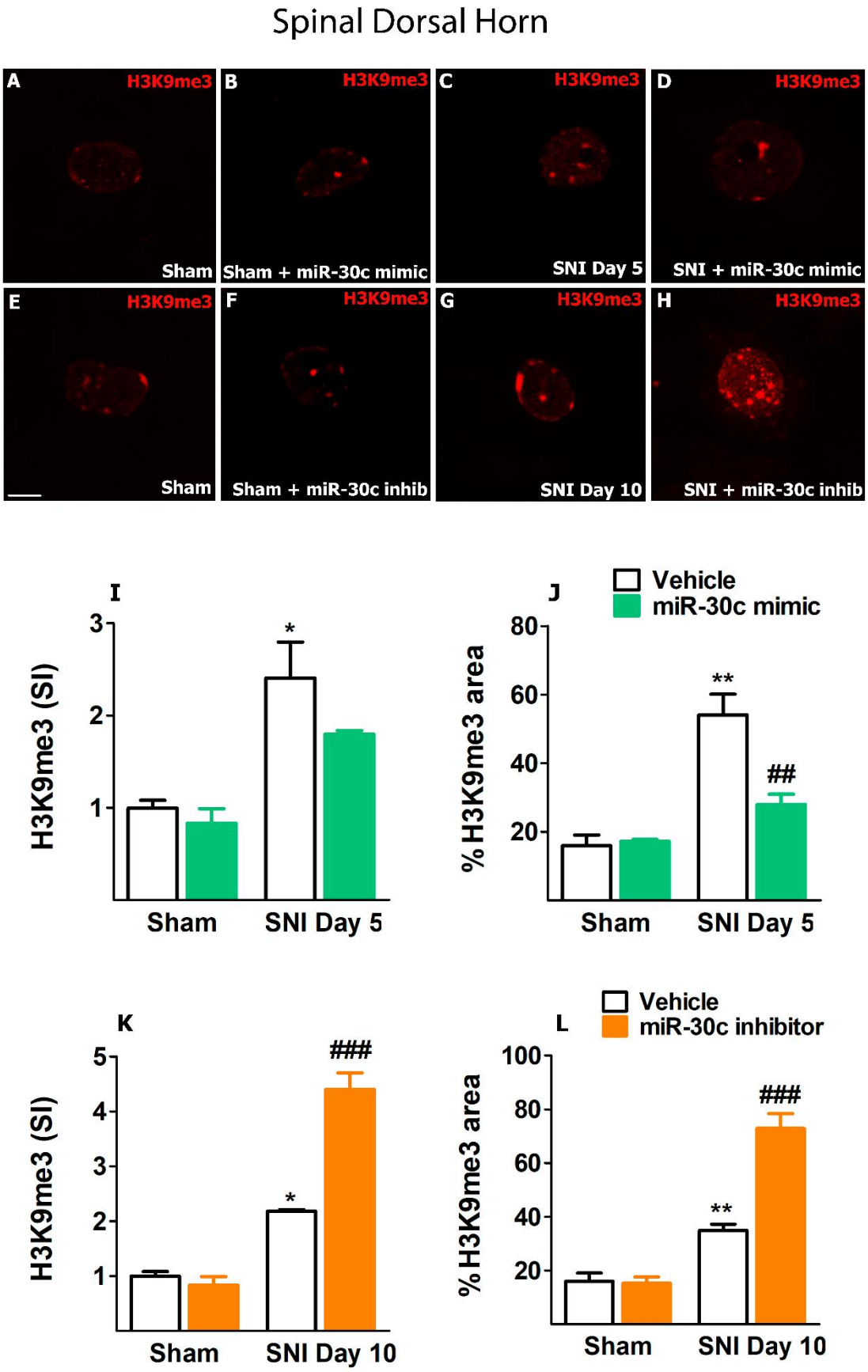

Dorsal Root Ganglia
A
H3K9me3
Sham
B
H3K9me3
Sham + miR-30c mimic
C
H3K9me3
SNI Day 5
D
H3K9me3
SNI + miR-30c mimic
E
H3K9me3
Sham
F
H3K9me3
Sham + miR-30c inhib
G
H3K9me3
SNI Day 10
H
SNI + miR-30c inhib
I
H3K9me3 (SI)
***
###
Sham
SNI Day 5
J
Vehicle
miR-30c mimic
% H3K9me3 area
***
##
Sham
SNI Day 5
K
H3K9me3 (SI)
###
Sham
SNI Day 10
Vehicle
miR-30c inhibitor
L
% H3K9me3 area
###
Sham
SNI Day 10

Figure 33 shows the cellular distribution of H3K9me3 in SDH neurons. SDH neurons from 5 days SNI-rats (**Fig. 33C**) showed an increase in the signal intensity (One-way ANOVA: $F_{(2, 8)} = 10.80$, $p<0.05$) and H3K9me3 methylated area (One-way ANOVA: $F_{(2, 8)} = 21.24$, $p<0.01$) that was significantly higher than sham-rats treated with vehicle (**Fig. 33A**). No significant changes were detected between 5 and 10 days SNI-rats, and in sham-rats, the treatment with miR-30c-5p mimic did not modify the H3K9me3 immunoreactivity (**Fig. 33B**).

SNI-rats treated with miR-30c-5p mimic (**Fig. 33D**) showed a slight but not significant decrease in the H3K9me3 signal intensity and a significant decrease in the H3K9me3 methylated area when compared to SNI-rats sacrificed on day 5 after the nerve injury (Two-way ANOVA: *H3K9me3 methylated area*: "treatment x nerve injury": $F_{(1, 7)} = 10.53$, $p<0.05$; "nerve injury": $F_{(1, 7)} = 33.46$, $p<0.001$; "treatment": $F_{(1, 7)} = 8.75$, $p<0.05$ (**Fig. 33I** and **J**).

SNI-rats sacrificed 10 days after nerve injury (**Fig. 33G**) also showed a significant increase in the signal intensity (One-way ANOVA: $F_{(2, 8)} = 10.80$, $p<0.05$) and H3K9me3 methylated area (One-way ANOVA: $F_{(2, 8)} = 21.24$, $p<0.01$) when compared with sham-rats treated with vehicle. In sham-rats, the treatment with miR-30c-5p inhibitor did not modify the H3K9me3 immunoreactivity (**Fig. 33E** and **F**).

Figure 32. Cellular localization and distribution pattern of H3K9me3 in dorsal root ganglion neurons from rats subjected to sciatic nerve injury and its modulation by miR-30c-5p mimic and miR-30c-5p inhibitor. Representative images showing H3K9me3-positive immunostaining in neurons isolated from the dorsal root ganglia (DRG) of sham-rats treated with vehicle (**A**) or miR-30c-5p mimic (**B**) SNI-rats sacrificed on day 5 treated with vehicle (**C**) or miR-30c-5p mimic (**D**), sham-rats treated with vehicle (**E**) or miR-30c-5p inhibitor (**F**) and SNI-rats sacrificed on day 10 treated with vehicle (**G**) or miR-30c-5p inhibitor (**H**). No positive signals are detected in DNA lacking areas such as the speckles. The average intensity of the signal (SI) (**I** and **K**) and the percentage of methylated area **(J** and **L)** were determined in 60 neurons (20 neurons per rat, 3 rats per group). ***p<0.001 vs Sham; ##p<0.01, ###p<0.001 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test). Scale bar: 5 μm.


Spinal Dorsal Horn
A
H3K9me3
Sham
B
H3K9me3
Sham + miR-30c mimic
C
H3K9me3
SNI Day 5
D
H3K9me3
SNI + miR-30c mimic
E
H3K9me3
Sham
F
H3K9me3
Sham + miR-30c inhib
G
H3K9me3
SNI Day 10
H
H3K9me3
SNI + miR-30c inhib
I
H3K9me3 (SI)
Sham
SNI Day 5
J
Vehicle
miR-30c mimic
% H3K9me3 area
Sham
SNI Day 5
K
H3K9me3 (SI)
Sham
SNI Day 10
L
Vehicle
miR-30c inhibitor
% H3K9me3 area
Sham
SNI Day 10

As observed in the DRG, SNI-rats treated with miR-30c-5p inhibitor exhibited a strong increase in the H3K9me3-immunoreactive signal and dense accumulations through all the nucleoplasm of SDH neurons (**Fig. 33H)**. The intensity of the fluorescent signal (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)}$ =41.07, p<0.001, "nerve injury": $F_{(1, 7)}$ =163.5, p<0.001; "treatment": $F_{(1, 7)}$ =30.66, p<0.001) and the H3K9me3 methylated area (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 7)}$ =24.68, p<0.01, "nerve injury": $F_{(1, 7)}$ =96.57, p<0.001; "treatment": $F_{(1, 7)}$ =22.7, p<0.01) were significantly higher after the treatment with miR-30c-5p inhibitor compared to SNI-rats treated with vehicle and sacrificed 10 days after the surgery (**Fig. 33K** and **L**)**.**

These findings further support the transcriptional repressive effect of the treatment with miR-30c-5p inhibitor in SNI-rats. In contrast, treating SNI-rats with miR-30c-5p mimic significantly decreased the percentage of H3K9me3-immunoreactivity area versus the SNI-rats treated with vehicle.

Figure 33. Cellular localization and distribution pattern of H3K9me3 in spinal dorsal horn neurons from rats subjected to sciatic nerve injury and its modulation by miR-30c-5p mimic and miR-30c-5p inhibitor. Representative images showing H3K9me3-positive immunostaining in neurons isolated from the spinal dorsal horn (SDH) of sham-rats treated with vehicle (**A**) or miR-30c-5p mimic (**B**), SNI-rats sacrificed on day 5 treated with vehicle (**C**) or miR-30c-5p mimic (**D**), sham-rats treated with vehicle (**E**) or miR-30c-5p inhibitor (**F**) and SNI-rats sacrificed on day 10 treated with vehicle (**G**) or miR-30c-5p inhibitor (**H**). No positive signals were detected in DNA lacking areas such as the speckles. The average intensity of the signal (SI) (**I** and **K**) and the percentage of methylated area (**J** and **L**) were determined in 60 neurons (20 neurons per rat, 3 rats per group). *p<0.05, ** p<0.01 vs Sham; ##p<0.01, ### p<0.001 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test). Scale bar: 5 μm.

Suv39h1 is a histone lysine methyltransferase that trimethylates Lys-9 of histone H3 using H3K9me1 as substrate. We evaluated whether the changes in the expression levels of Suv39h1 in the DRG and SDH paralleled those observed in H3K9me3 after the treatment with miR-30c-5p inhibitor or miR-30c-5p mimic. Suv39h1 levels showed a significant increase in SNI-rats sacrificed 5 days after the surgery in the DRG (**Fig. 34A**) and the SDH (**Fig. 34C**). Suv39h1 levels also significantly decreased in the DRG and SDH of 10 days-SNI rats when compared to 5 days SNI-rats [DRG: (One-way ANOVA: $F_{(2, 13)}$ =8.99, p<0.01; *SNI Day 5 vs. sham* p<0.01; *SNI Day 5 vs. SNI Day 10* p<0.05). SDH: (One-way ANOVA: $F_{(2, 15)}$ =7.31, p<0.01; *SNI Day 5 vs. sham* p<0.05; *SNI Day 5 vs. SNI Day 10* p<0.05)]. In SNI-rats treated with miR-30c-5p mimic, Suv39h1 levels showed a significant decrease in the DRG (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 12)}$ =9.87, p<0.01, "nerve injury": $F_{(1, 12)}$ =5.48, p<0.05; "treatment": $F_{(1, 12)}$ =6.79, p<0.05) (**Fig. 34B**) and the SDH (Two-way ANOVA: "nerve injury": $F_{(1, 16)}$ =7.14, p<0.05) (**Fig. 34C**) when compared with SNI-rats sacrificed 5 days after the surgery. In sham-rats, the treatment with miR-30c-5p mimic did not alter the Suv39h1 expression levels compared to those treated with vehicle (**Fig. 34A** and **C**). Accordingly, with the immunofluorescence data on histone H3K9 trimethylation, the expression levels of Suv39h1 increased significantly in both DRG (Two-way ANOVA: "nerve injury": $F_{(1, 15)}$ =4.55, p<0.05) and SDH (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 12)}$ =4.974, p<0.05) from SNI-rats treated with miR-30c-5p inhibitor (**Fig. 34B** and **D**). Sham-rats treated with miR-30c-5p inhibitor did not show significant differences in the expression levels of Suv39h1 either in the DRGs or the SDH compared to sham-rats treated with vehicle.

Our results indicate that the protection against neuropathic pain provided by the treatment with miR-30c-5p inhibitor to SNI-rats was associated with a global inhibition of the genetic transcription through DNA and histone hyper-methylation in pain-related structures.

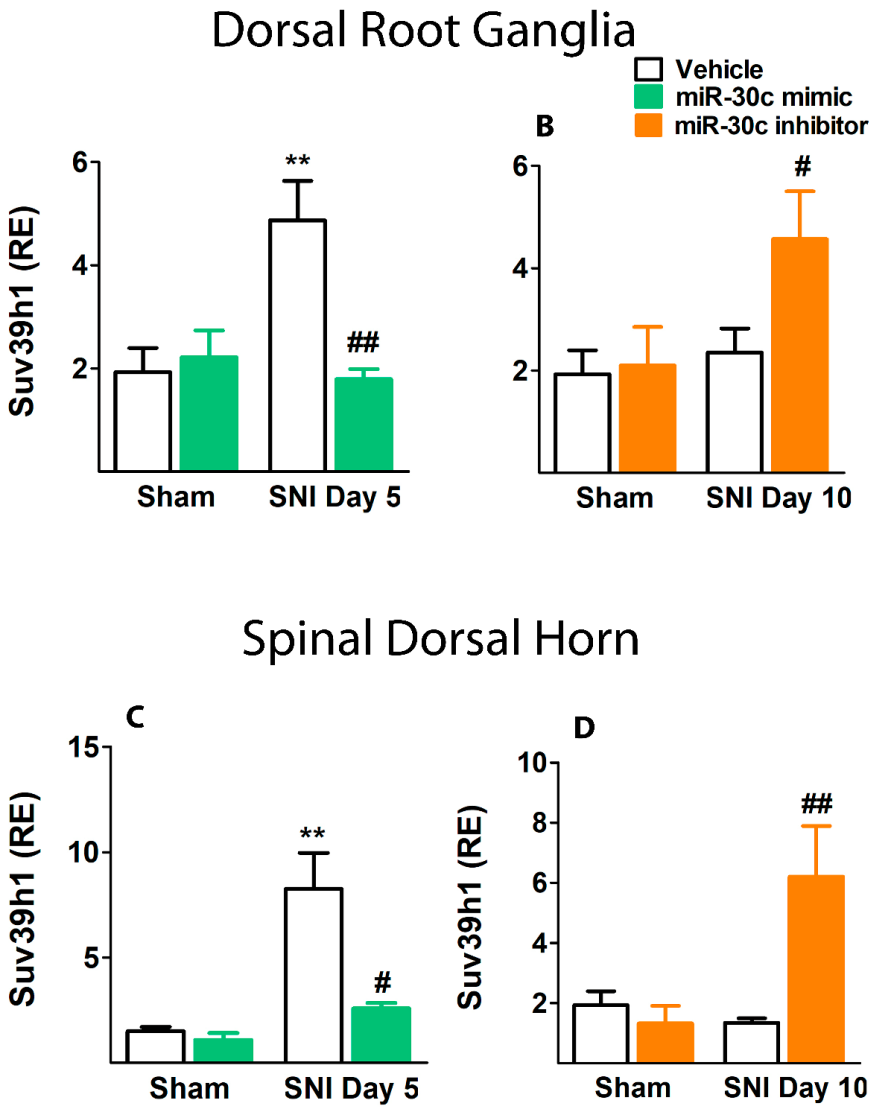


Figure 34. Effects of the treatment with miR-30c-5p mimic or miR-30c-5p inhibitor on the expression of Suv39h1 in dorsal root ganglion and spinal dorsal horn from rats subjected to sciatic nerve injury. Relative expression levels of Suv39h1 in the dorsal root ganglia (DRG) (**A** and **B**) and spinal dorsal horn(SDH) (**C** and **D**) from sham and SNI-rats treated with vehicle, miR-30c-5p inhibitor or miR-30c-5p mimic (Sham: n=6; Sham + miR-30c-5p mimic: n=5; Sham + miR-30c-5p inhibitor: n=5; SNI Day 5: n=8; SNI Day 10: n=8; SNI + miR-30c-5p inhibitor: n=6; SNI + miR-30c-5p mimic: n=6). **p<0.01 vs Sham; # p<0.05, ## p<0.01 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test).

2. Morphologic, ultrastructural, and functional changes induced by miR-30c-5p modulation in primary sensory neurons after sciatic nerve injury

While performing the histological experiments which result has been previously described, we realized that neurons from the DRGs of SNI and SNI treated with miR-30c-5p mimic animals presented alterations in the cytoplasm that suggested chromatolytic damage and thus alterations in the protein synthesis machinery. These findings led us to hypothesize that nerve injury and miR-30c-5p overexpression after SNI would result in a deregulation of genes involved in such crucial processes for neuronal survival , which might hinder the recovery of injured sciatic neurons and intensify neuropathic pain. Therefore, we further assessed the morphologic, ultrastructural, and functional consequences of miR-30c-5p modulation in DRG neurons after SNI in rats.

2.1 Effects of miR-30c-5p modulation on the protein synthesis machinery in dorsal root ganglion neurons after sciatic nerve injury

The ribosome is a complex of ribosomal RNAs (rRNAs) and proteins that uses transfer RNAs (tRNAs) and amino acids to synthesize proteins from mRNAs. Ribosome biogenesis is a tightly organized multistep process, during which ribosomal proteins are synthesized in the cytoplasm and immediately imported to the nucleolus where they are assembled with rRNA into the pre-ribosome. The nearly complete ribosomal subunits are exported back to the cytoplasm for the final steps of assembly.

The Nissl bodies are constituted by parallel arrays of cisterns of rough endoplasmic reticulum (RER) studded with ribosomes and associations of free polyribosomes. Nissl bodies are a major component of the protein synthesis machinery of a neuron. Axotomy, as well as other injuries, such as ischemia stress, toxics, etc., can trigger the phenomenon termed chromatolysis (fragmentation of Nissl substance), which is characterized by the fragmentation of the rough endoplasmic reticulum leaving clear areas of cytoplasm lacking Nissl bodies. This can be accompanied by degranulation, disaggregation and/or disassembly of polyribosomes and degradation of

monoribosomes. Chromatolysis leads to apoptosis unless a regeneration process is started (Stoica and Faden, 2010).

We explored the occurrence of alterations in the Nissl substance distribution and integrity under our experimental conditions. **Figure 35** shows representative images of isolated L4, L5 and L6 DRG neurons stained with the nucleic acid marker propidium iodide (PI). The neurons from sham-rats treated with vehicle (**Fig. 35A** and **E**), miR-30c-5p mimic (**Fig. 35B**) or miR-30c-5p inhibitor (**Fig. 35F**), presented the reference normal distribution of ribosomes and Nissl bodies within the neuronal perikaryon. The injured DRG neurons from SNI-rats sacrificed on day 5 (**Fig. 35C**) or 10 (**Fig. 35G**) after SNI presented an intense central chromatolytic process. The Nissl bodies underwent dissolution and any remaining ribonucleoprotein complexes shifted from the center to the periphery of the cell body, leaving an almost empty cytoplasm. The DRG neurons from SNI-rats treated with miR-30c-5p mimic (**Fig.35D**) presented chromatolysis even more intense than SNI-rats. In contrast, the density and distribution of the Nissl substance in DRG neurons from SNI-rats treated with miR-30c-5p inhibitor recovered a pattern similar to the control neurons (**Fig. 35H**).

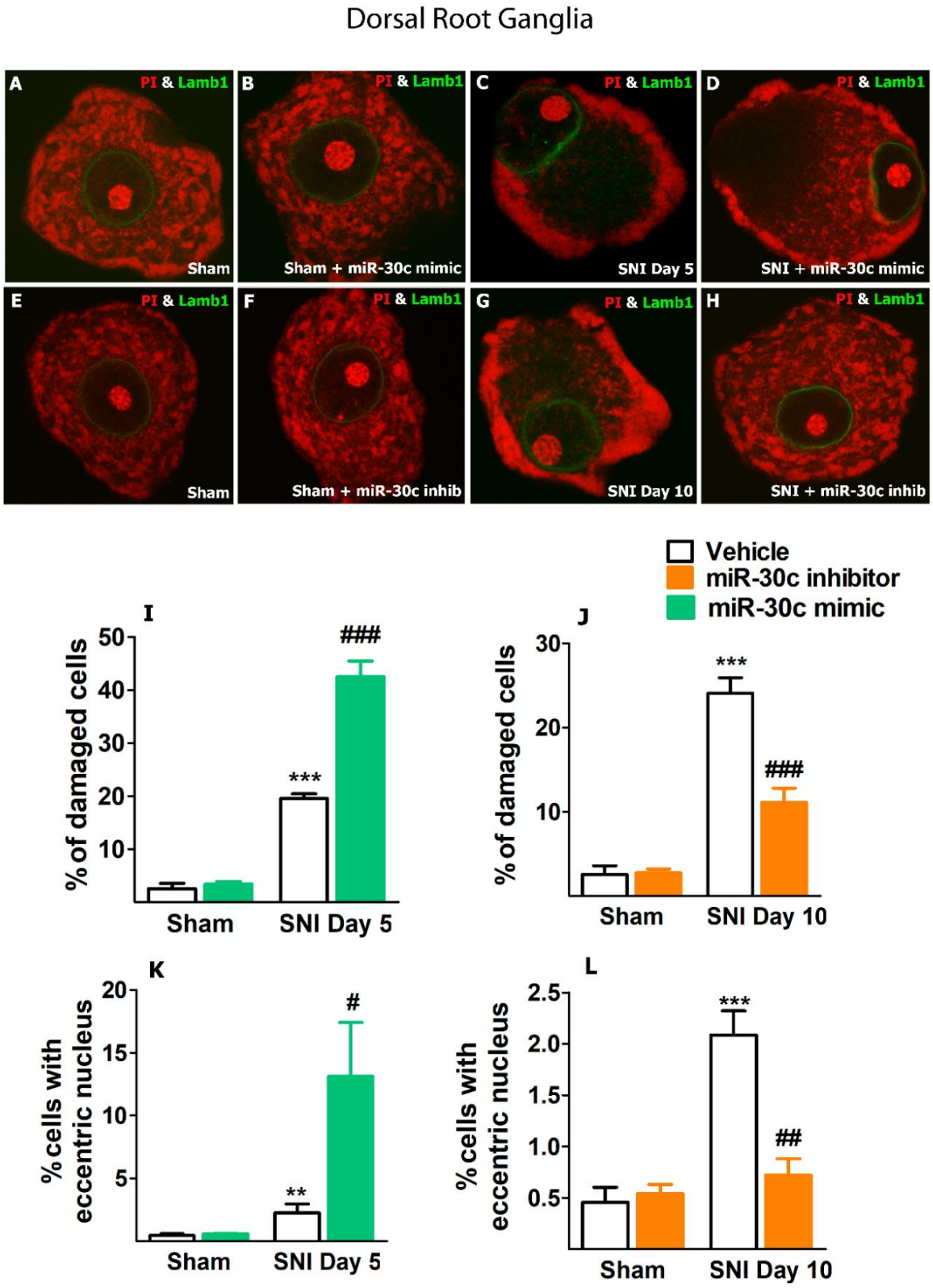
The percentage of damaged neurons (**Fig. 35I** and **J**) was determined in 1,000 neurons per rat (n = 3 rats per group). Almost 25% of the DRG neurons from SNI-rats showed chromatolytic damage (5 days-SNI + vehicle: 19.5±0.86 %; 10 days-SNI + vehicle: 24.1±1.95 %; One-way ANOVA: $F_{(2, 8)} = 77.38$, $p<0.001$). The treatment with miR-30c-5p mimic significantly increased the percentage of chromatolytic neurons (5 days-SNI + miR-30c-5p mimic: 42.5±2.95 %; Two-way ANOVA: "treatment x nerve injury": $F_{(1, 8)} = 45.83$, $p<0.001$, "nerve injury": $F_{(1, 8)} = 293.6$, $p<0.001$; "treatment": $F_{(1, 8)} = 56.61$, $p<0.001$). On the other hand, the presence of chromatolytic neurons was significantly reduced by the treatment with miR-30c-5p inhibitor (10 days-SNI + miR-30c-5p inhibitor: 11.1±1.67 %; Two-way ANOVA: "treatment x nerve injury": $F_{(1, 8)} = 23.83$, $p<0.01$, "nerve injury": $F_{(1, 8)} = 123.5$, $p<0.001$; "treatment": $F_{(1, 8)} = 22.61$, $p<0.01$).

Displacement of the nucleus from its central position to the periphery is considered other chromatolysis-related change. Immunofluorescence with an

antibody against Lamin B1 revealed that the nuclei of DRG neurons from sham-rats treated with vehicle (**Fig. 35A**), miR-30c-5p mimic (**Fig. 35B**) or miR-30c-5p inhibitor (**Fig. 35F**), as well as from SNI-rats treated with miR-30c-5p inhibitor (**Fig. 35H**) presented the normal smooth and rounded shape, and were located in the middle of the cells. In contrast, compared to control neurons, the nuclei of some DRG neurons from SNI-rats and, particularly, from SNI-rats treated with miR-30c-5p mimic (**Fig. 35C, D** and **G**), were displaced to an eccentric position in the cell, presented a bigger size and more oval shape, and exhibited numerous folds in the nuclear envelope, all of which are typical features of chromatolysis.

The proportion of neurons with eccentric nuclei increased significantly in SNI-rats treated with miR-30c-5p mimic (13.1±4.3 %; Two-way ANOVA: "nerve injury": $F_{(1, 7)}$ =8.39, $p<0.05$;) in comparison with SNI-rats treated with vehicle (2.27±0.70 %) (**Fig. 35K** and **L**).

Figure 35. Effects of modulation of miR-30c-5p on the chromatolysis developed by dorsal root ganglion neurons after sciatic nerve injury. Representative images of double staining for RNA [propidium iodide (PI), red)] and Lamin B1 (LamB1, green) (**A** to **H**) in dissociated dorsal root ganglion (DRG) neurons. **A, B, E** and **F**: The neurons from sham-rats show the normal structural features anddistribution pattern of the Nissl bodies and nuclei. The neurons from SNI-rats sacrificed 5 (**C**) and 10 (**G**) days after the surgery and from SNI-rats treated with miR-30c-5p mimic (**D**) present central chromatolysis features such as reduction and dispersion of the Nissl bodies from the center of the neuron body towards the plasmatic membrane, and displacement of the nucleus towards the periphery of the perikaryon. **H**: The neurons from SNI-rats treated with miR-30c-5p inhibitor exhibit similar structural features than those from sham-rats. **I** and **J**: Percentage of neurons showing chromatolytic morphology. **K** and **L**: Percentage of neurons showing eccentricity of the nucleus. The percentage of damaged neurons and eccentric nucleus were determined in 1,000 neurons per rat (n=3 rats per group). **p<0.01, ***p<0.001 vs Sham; #p<0.05, ## p<0.01, ###p<0.001 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test). Scale bar: 5 μm.



The treatment with miR-30c-5p inhibitor (**Fig. 35H**) prevented the chromatolytic alterations observed after the SNI in rats. Thus, less than 2% of neurons from SNI-rats treated with miR-30c-5p inhibitor showed nuclear

eccentricity (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 6)}$ =14.50, p<0.01, "nerve injury": $F_{(1, 6)}$ =22.6, p<0.01; "treatment": $F_{(1, 8)}$ =11.36, p<0.05) (**Fig. 35K** and **L**).

Neither the treatment with miR-30c-5p mimic nor miR-30c-5p inhibitor produced any obvious change in sham-rats (**Fig. 35B** and **F**).

We further analyze, using transmission electron microscopy, the ultrastructural features of SNI-induced chromatolysis in DRG neurons and its modulation by miR-30c-5p interference. DRG neurons from both sham-rats (**Fig. 36A**) and SNI-rats treated with miR-30c-5p inhibitor (**Fig. 36C**) exhibited the typical arrangement of the Nissl bodies composed of aggregates of polyribosomes and RER cisterns (**Fig. 36C, arrow**), well distributed though the cytoplasm. Inside the Nissl bodies, free polyribosomes were also observed (**Fig. 36A, arrow**). Severe structural alterations were observed in the DRG neurons from SNI (**Fig. 36B**) and, especially, from SNI-rats treated with miR-30c-5p mimic (**Fig. 36D**). Extensive RER-poor chromatolytic areas were observed in all the cytoplasm, indicating a severe disruption of protein synthesis machinery. In addition, we observed an increase in the number and size of mitochondria that also suggests alterations in the energy machinery under the neuropathic pain condition.

Overall, these results indicate that neuropathic pain after SNI associated severe dysfunctions in the protein synthesis machinery, which are potentiated by the treatment with miR-30c-5p mimic. Moreover, inhibition of miR-30c-5p constituted an efficient neuroprotective mechanism to reduce neuronal damage after SNI, which might contribute to prevent neuropathic pain development.

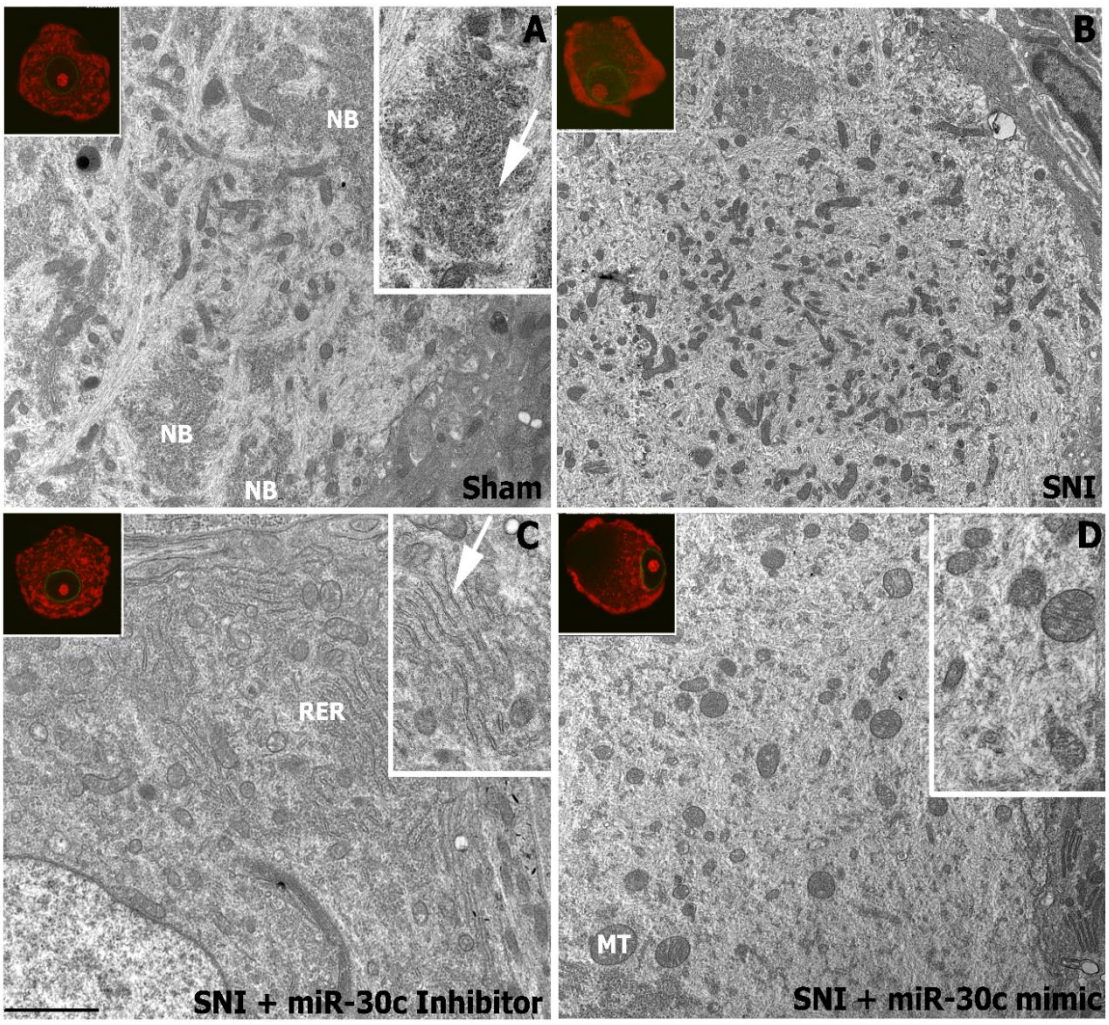


Figure 36. Ultrastructural characteristics of dorsal root ganglion neurons. Representative electronic micrographs of the central zone of the cytoplasm from sham-rats (**A**), SNI-rats (**B**), SNI-rats treated with miR-30c-5p inhibitor (**C**) and SNI-rats treated with miR-30c-5p mimic (**D**). In DRG neurons from sham rats (**A**) and SNI-rats treated with miR-30c-5p inhibitor (**C**) the most prominent organelles are the Nissl bodies (NB) composed of cisternae of rough endoplasmic reticulum (RER) (**C** detail) and rosettes of free poliribosomes (**A** detail). Profiles of Golgi complexes and mitochondria are also apparent. Upon SNI (**B**) and SNI plus miR-30c-5p mimic treatment (**D**) the Nissl bodies disappear (chromatolysis) and the central cytoplasm is of a pale aspect and contains prominent mitochondria (MT). Scale bar: 5 μm.

2.2 Effects of miR-30c-5p modulation on the nucleolar cytoarchitecture of dorsal root ganglion neurons after sciatic nerve injury

The nucleolus is a nuclear factory for the synthesis of rRNAs, processing of rRNA transcripts, and assembly of the pre-ribosomal subunits. These processes are intrinsically regulated to achieve the adaptation of proteostasis to the actual cellular translation demands. The level of activation of the

nucleolus is reflected in the structural organization and distribution of its components: the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC). In addition, some studies support a function for the nucleolus as a cellular sensor that detects and coordinates the cellular response to stress (Boulon *et al*., 2010).

Since ribosome biogenesis is an essential step to sustain protein synthesis activity, we analyzed the response of the nucleolus to severe chromatolysis in DRG of SNI-animals and the impact of miR-30c-5p gain and loss of function in the organization and function of this organelle. To determine whether the dysfunction of the protein synthesis caused by the severe chromatolysis modified the nucleolar architecture in DRG neurons, we performed a morphometric and quantitative study of the nucleolar components in DRG neuronal dissociates. Several studies show that the number and organization of the FC in neurons correlate with the transcriptional activity of the rDNA (Berciano *et al*., 2007; Hernández-Verdún *et al*., 2010). The FC concentrates components of the RNA polymerase I transcription machinery, such as the upstream binding factor (UBF) and RNA polymerase I, involved in rRNA gene transcription (Lafarga *et al*., 2017). Immunolabeling for UBF allows to define the number, structure and distribution of the FCs. These parameters are positively related to the transcriptional activity of the nucleolus (Berciano *et al*., 2007; Hernández-Verdún *et al*., 2010).

Our results of double immunofluorescence staining for Lamin B1 and UBF in DRG neurons from sham-rats treated with vehicle, miR-30c-5p mimic or miR-30c-5p inhibitor showed the typical reticulated UBF immunoreactivity pattern of neurons with high transcriptional activity, with numerous small-sized UBF positive spots distributed throughout the nucleolar body (**Fig. 37A, B, E** and **F**). On the other hand, 5 and 10 days SNI-rats (**Fig. 37C** and **G**) and specially SNI-rats treated with miR-30c-5p mimic (**Fig. 37D**) exhibited a profound reorganization of the FCs, which included their enlargement and marginal segregation in several large masses within the nucleolar body. On the other hand, SNI-rats treated with miR-30c-5p inhibitor (**Fig. 37H**) showed a pattern of UBF immunoreactivity similar to sham-rats. Moreover, a quantitative analysis of the neurons showing UBF-positive nuclear spots (Fig. 37I and J) revealed that DRG neurons from both SNI-rats treated with vehicle

(5 days SNI + vehicle: 20.7±0.86 %; 10 days SNI + vehicle: 24.1±1.95 %; One-way ANOVA: $F_{(2, 8)}$ =14.73, p<0.01; *SNI Day 5 vs. sham* p<0.05; *SNI Day 10 vs. sham* p<0.01) and SNI-rats treated with miR-30c-5p mimic (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 8)}$ =56.39, p<0.001, "nerve injury": $F_{(1, 8)}$ =194.5, p<0.001; "treatment": $F_{(1, 8)}$ =38.82, p<0.001) presented high rates of nucleolar macrosegregation (20.7 ± 3.99 % and 50.6 ± 0.92 %, respectively) when compared with sham rats-treated with vehicle (6.7 ± 1.13 %) or with miR-30c-5p mimic (3.89 ± 1.67 %).

In contrast, DRG neurons from SNI-rats treated with miR-30c-5p inhibitor (8.0 ± 2.12 %) presented nucleolar macrosegregation rates lower than SNI-rats treated with vehicle (Two-way ANOVA: "treatment x nerve injury": $F_{(1, 8)}$ =26.68, p<0.001, "nerve injury": $F_{(1, 8)}$ =56.47, p<0.001; "treatment": $F_{(1, 8)}$ =44.45, p<0.001), and similar to those of sham-rats treated with vehicle. In sham rats, the treatment with miR-30c-5p or miR-30c-5p inhibitor did not change the nucleolar organization in DRG neurons.

The ultrastructural organization of the nucleolus was further analyzed by transmission electron microscopy. FCs appeared as numerous small-sized circular areas surrounded by a ring of DFC. The nucleolar components in DRG neurons from sham-rats and SNI-rats treated with miR-30c-5p inhibitor presented the normal structure and distribution of FCs (**Fig. 38A** and **C**). On the other hand, a profound segregation and reorganization of the nucleolar components was observed in many DRG neurons from both SNI-rats treated with vehicle (**Fig. 38B**) and SNI-rats treated with miR-30c-5p mimic (**Fig. 38D**). These changes were accompanied by other nucleolar alterations, such as the segregation of large masses of GC and the formation of nucleolar cavities.

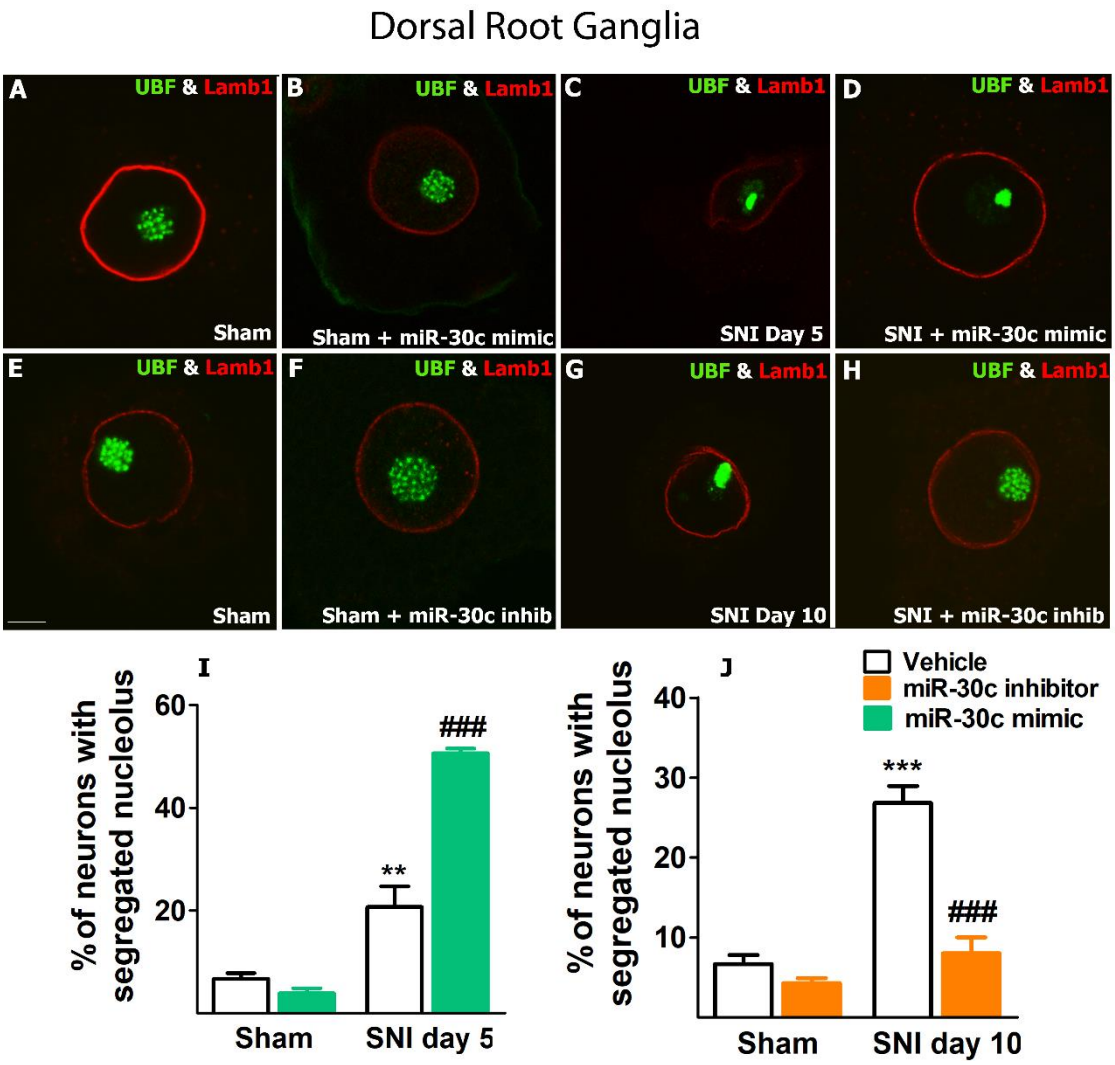


Figure 37. Effects of miR-30c-5p modulation on the nucleolar cytoarchitecture of dorsal root ganglion neurons after sciatic nerve injury. **A** to **G**: Representative immunofluorescence staining for Lamin B1 (LamB1, red) and upstream binding factor (UBF, green) in DRG neurons from sham-rats treated with vehicle (**A** and **E**), sham-rats treated with miR-30c-5p mimic (**B**), 5 days-SNI-rats treated with vehicle (**C**), 5 days-SNI-rats treated with miR-30c-5p mimic (**D**), sham-rats treated with miR-30c-5p inhibitor (**F**), 10 days-SNI rats treated with vehicle (**G**), and 10 days-SNI-rats treated with miR-30c-5p inhibitor (**H**). The normal distribution of UBF in small dots is observed in **A, B, E, F** and **H**. Representative DRG neurons with macrosegregation of the components of the nucleolus are shown in **C, D** and **G**. **H** and **I:** The percentage of neurons showing nucleolar segregation was determined in 100 neurons per rat (n = 3 rats per group).; ** p<0.01, *** p<0.001 vs Sham; ### p<0.001 vs SNI (Two-way ANOVA followed by Bonferroni post hoc test). Scale bar: 5 μm.

Our results indicate that SNI induced important structural alterations of the nucleolus in the DRG primary neurons, in association with neuropathic pain

development. The harmful effect of SNI was potentiated by the treatment with miR-30c-5p mimic, with pro-allodynic consequences. In contrast, SNI-induced DRG damage was prevented by the treatment with miR-30c-5p inhibitor with anti-allodynic consequences.

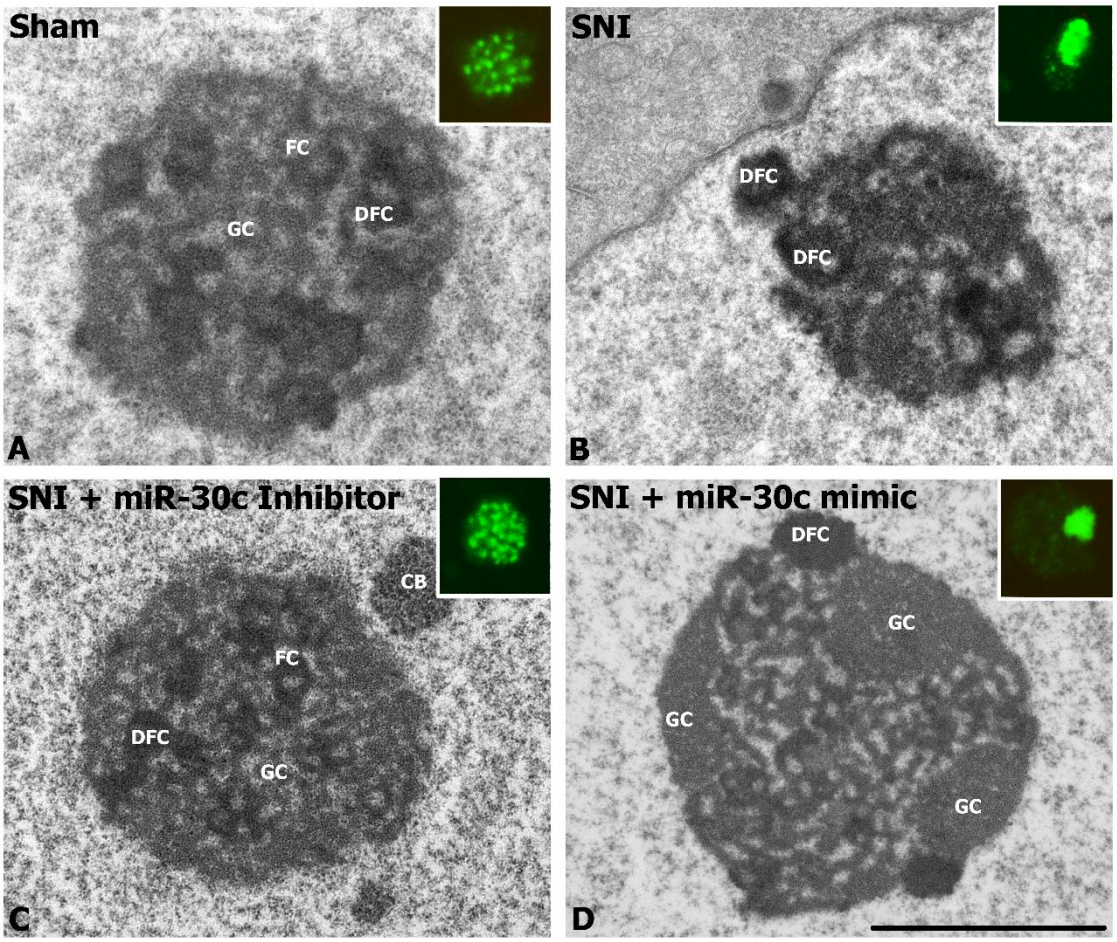


Figure 38. Effects of miR-30c-5p modulation on the nucleolar ultrastructure of dorsal root ganglion neurons after sciatic nerve injury. Representative electron micrographs and representative immunofluorescence staining for upstream binding factor (UBF, green) in DRG neurons from sham-rats (**A**), SNI-rats treated with vehicle (**B**), SNI-rats treated with miR-30c-5p inhibitor (**C**) and SNI-rats treated with miR-30c-5p mimic (**D**). Sham rats (**A**) and SNI-rats treated with miR-30c-5p inhibitor (**C**) exhibit the normal nucleolus characterized by a reticulated configuration composed of numerous fibrillar centers (FCs) surrounded by a thin shell of dense fibrillar component (DFC) embedded in the granular component (GC). SNI-rats treated with vehicle (**B**) and SNI-rats treated with miR-30c-5p mimic (**D**) present severe nucleolar alterations including the formation of enlarged FCs, segregation of large masses of GC and the formation of nucleolar cavities. Scale bar: 2 um.-

2.3 Effects of miR-30c-5p modulation on the Cajal bodies of dorsal root ganglion neurons after sciatic nerve injury

The Cajal Body (CB) is a multifunctional organelle whose number and size are directly related with the transcriptional activity of the cell (Lafarga *et al*.,2017; Pena *et al*.,2001; Berciano *et al*.,2007), which is physically and functionally related to the nucleolus. As mentioned in the introduction, one of the most important functions of the CBs is related to the biogenesis and recruitment of the snRNPs and snoRNPs, proteins required for the processing of the immature 45S pre-rRNA in the DFC of the nucleolus (Verheggen *et al*., 2001; Machyna *et al*., 2013). Therefore, the next step of our study was to analyze the behavior of the CBs in response to SNI and the effects of miR-30c-5p modulation. We determined the number and organization of the CBs in dissociated DRG neurons immunostained for coilin, a structural mark of the canonical CBs. Coilin immunolabeled CBs appeared as sharply defined round nuclear bodies, frequently attached to the nucleolus. **Figure 39** shows DRG neurons exhibiting 0 (**A**), 1 (**B**), 2 (**C**) and even 5 (**D**) CBs.

In all the experimental groups, most of the neurons showed only one CB. The treatment of SNI-rats with miR-30c-5p inhibitor induced a significant increase in the number of CBs compared with SNI-rats treated with vehicle. Thus, many neurons from SNI-rats treated with miR-30c-5p inhibitor presented more than two CBs (28.84 ± 2.37 %) (One-way ANOVA: $F_{(3, 10)} = 52.67$, $p<0.001$: SNI Day 10 vs. SNI + miR-30c inhibitor $p<0.001$). Interestingly, 22.09 ± 1.37 % of the neurons of SNI-rats and a 18.29± 5.86 % of the neurons of SNI-rats treated with miR-30c-5p mimic did not present any CB (One-way ANOVA: $F_{(3, 11)} = 13.50$, $p<0.01$: SNI Day 10 vs. sham $p<0.01$; SNI + miR-30c mimic vs sham $p<0.05$, SNI Day 10 vs. SNI + miR-30c inhibitor $p<0.01$).

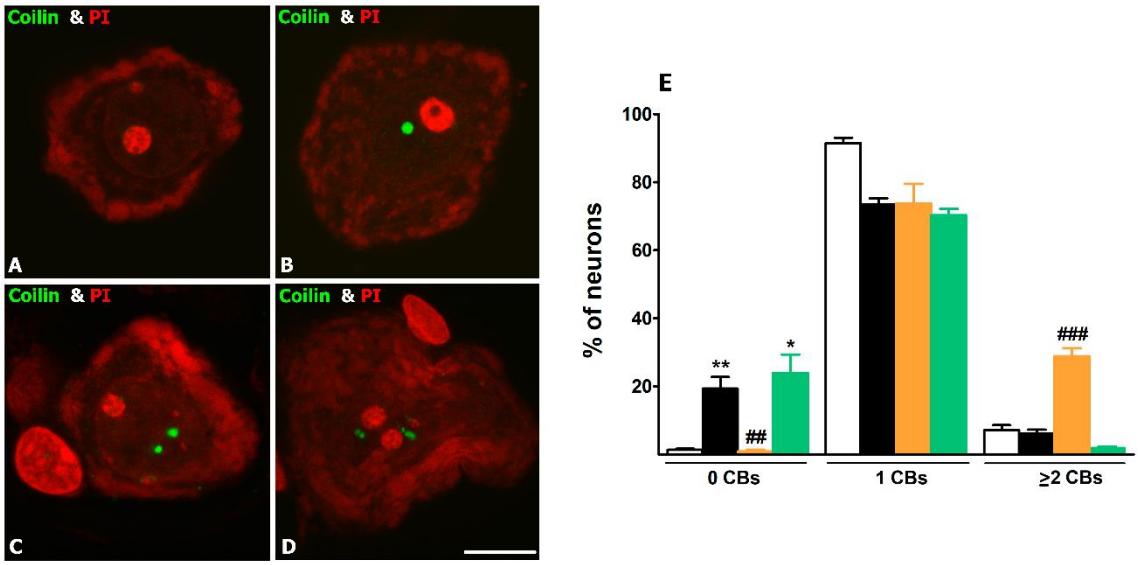


Figure 39. Effects of miR-30c-5p modulation on the Cajal bodies of dorsal root ganglion neurons after sciatic nerve injury. Representative images of double staining for RNA [propidium iodide (PI), red)] and coilin (green) (**A** to **D**) in dissociated dorsal root ganglion (DRG) neurons. Example of neurons showing 0 (**A**), 1 (**B**), 2 (**C**) and 5 (**D**) CBs. The number of CBs per neuron was determined in 100 neurons per rat, in 3 rats of each group (sham; SNI + vehicle; SNI + miR-30c inhibitor; SNI + miR-30c mimic). **E**: The quantification analysis indicate that, regardless the experimental condition, most neurons present 1 CB. There is a significant increase in the percentage of neurons showing more than 2 CBs in SNI-rats treated with miR-30c-5p inhibitor. The proportion of neurons without CBs is significantly increased in SNI-rats treated with miR-30c-5p mimic. ***p<0.001; ## p<0.01; ###p<0.001). Two-tailed Student's *t*-test. Scale bar: 5 μm.

The ultrastructural analysis by electron microscopy confirmed that after the treatment with miR-30c-5p inhibitor the CBs were hypertrophic and closely positioned to the nucleolus (**Figure 40**).

This results could contribute to the neuroprotective anabolic response to normalize the alterations that were produced by the nerve injury. Inhibition of miR-30c protects against nucleolus disruption and promotes an increase in the rate of maturation of the immature pre-rRNA that is also reflected in an increase in the number of CBs. With these neuroprotective responses, the neuron is ready to synthetize and mature more ribosomes to fulfill the requirements of the cell to survive. On the other hand, SNI and specially SNI + miR-30c mimic rats showed segregated nucleoli and reduced number of CBs per neuron which might reflect a global disruption in the protein synthesis.

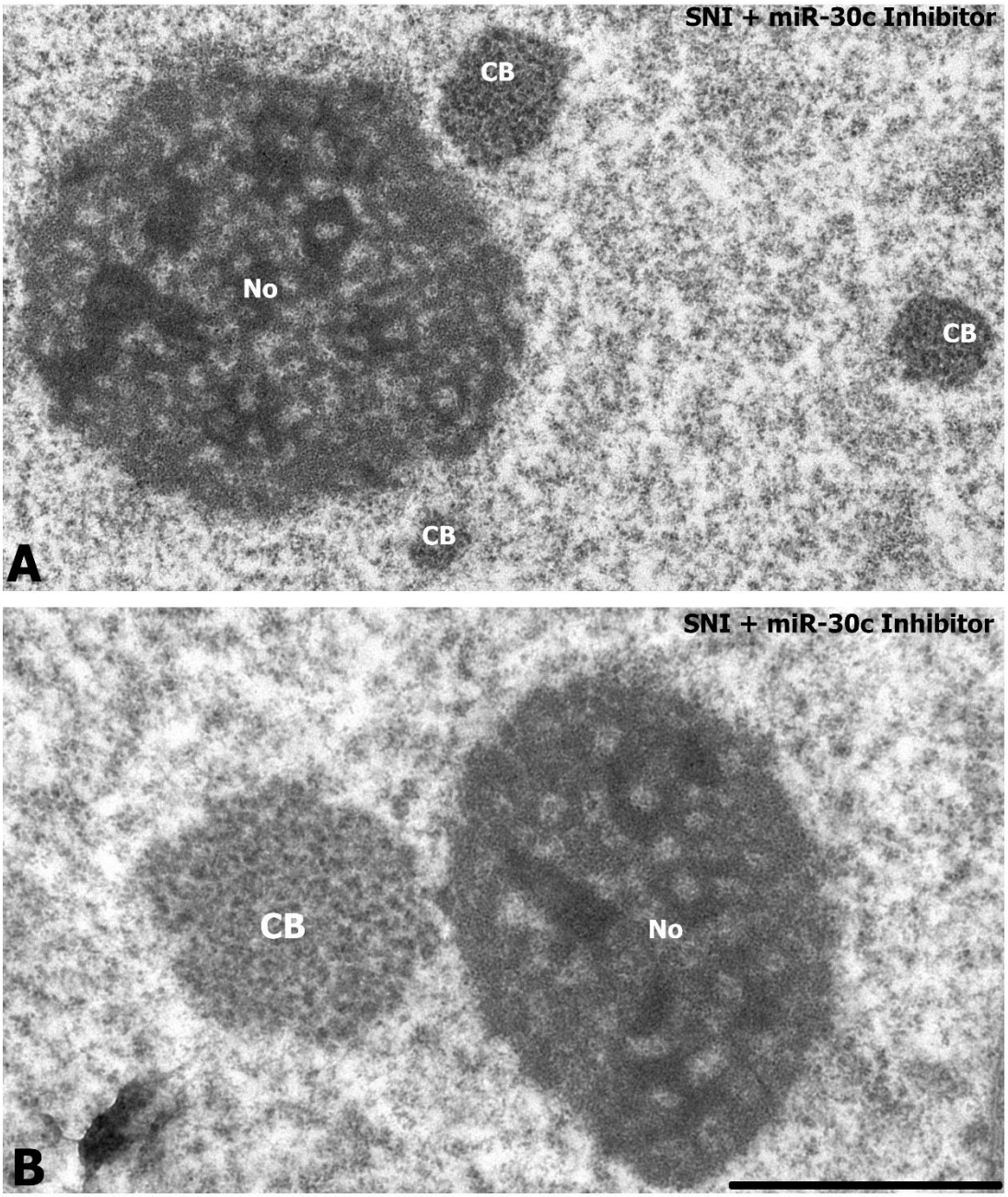


Figure 40. Electron microscopy study of the Cajal Bodies in DRG neurons from SNI rats treated with miR-30c-5p inhibitor showing 3 Cajal Bodies (CBs) (**A**) and a hypertrophic CB physically close to the nucleolus (No) (**B**). Scale bar: 2 µm.

Discussion

Acute pain is an evolutionary mechanism of defense triggered by the sensory nervous system to protect the body from actual or potential damaging processes. In some pathological conditions, pain can persist for months or even years beyond healing time. Chronic pain is now considered a disease entity in and of itself, which constitutes a serious burden for the daily life of patients. It is the most frequent reason of medical care demand and generates a great impact in clinical and socio-economic terms (Mills *et al*., 2016).

Neuropathic pain is a type of chronic pain that arises from diseases or lesions of the somatosensory nervous system (IASP). Its etiology is multiple; it can appear after traumatic injuries, chronic ischemia, viral infections, metabolic diseases, tumoral processes, stroke, etc. (Gilron *et al*., 2015). After the healing of the neural lesion, some individuals develop a painful and debilitating syndrome characterized by exaggerated responses to painful stimuli (hyperalgesia), dysesthetic pain, spontaneous and/or triggered by normally innocuous stimuli (allodynia), which can persist for very long periods (Cerveró *et al*.,2009; Baron *et al*.,2010). Chronic neuropathic pain is a major health problem affecting up to 10% of the general population (Goldberg and McGee, 2011) and it is poorly controlled by currently available pharmacological treatments and non-pharmacological interventions. Moreover, side effects limit frequently the use of anti-neuropathic drugs, especially in vulnerable patients (Finnerup *et al*., 2015).

Epigenetic modifications, such as DNA methylation, histone modifications and miRNAs, have been increasingly implicated in the long-lasting aberrant expression of crucial pain-related genes in the somatosensory nervous system, underlying pain chronification after neural injuries (Graff *et al*., 2011; Bucheit *et al*., 2012; Lutz *et al*., 2012; Rahn *et al*., 2013; Karpova, 2014; Mauck *et al*., 2014; Wang *et al*., 2014; Descalzi *et al*., 2015; Niederberger *et al*., 2017; Penas and Navarro, 2018).

In this regard, over the last years, our group is dealing with the role of miRNA-related epigenetic mechanisms involved in the persisting neural adaptations triggered by peripheral nerve damage, which contribute to the development of neuropathic pain. Our previous results (Tramullas *et al*., 2018) support a relevant role for miR-30c-5p in neuropathic pain

development and maintenance in rodent models of nerve injury and in patients with critical leg ischemia suffering from neuropathic pain. This study opens novel therapeutic opportunities to interfere with chronic pathological pain establishment and supports the value of circulating miR-30c-5p as potential biomarker of neuropathic pain (Tramullas *et al*., 2018).

In this Doctoral Thesis, our objectives were to analyze in greater depth the epigenetic mechanisms activated by SNI to trigger neuropathic pain, and to uncover the participation of miR-30c-5p.

Our starting point was to induce a traumatic peripheral neuropathy to validate the effects of miR-30c-5p-gain and -loss of function in pain-related behaviours of rats, using the model of spared nerve injury (Decosterd and Wolf, 2000). The rats subjected to SNI presented hyperalgesia and allodynia evoked by mechanical stimuli applied to the territory innervated by the sural branch of the sciatic nerve (lateral region of the plantar surface), whose integrity was preserved after spared nerve injury. Allodynia became evident from the 5th day of the SNI, reached the maximum severity 10 days after SNI, and persisted stable over the follow-up. Consisting with our previous findings (Tramullas *et al*., 2018), the present results show that a short early cycle of three intracisternal injections of miR-30c-5p inhibitor prevented the development of neuropathic pain after SNI in rats, for the complete follow-up period. In contrast, the treatment with miR-30c-5p mimic accelerated and intensified neuropathic pain.

Both the prolonged (>6 months) behavioral modifications that induces SNI (Decosterd and Wolf, 2000) and the sustained antiallodynic effect produced by the administration of only three injections of the miR-30c-5p inhibitor (present results and Tramullas *et al*., 2018) support the contribution of long-lasting epigenetic mechanisms in both experimental settings.

DNA methylation, which associates with heterochromatin formation and transcription silencing, constitutes a major epigenetic mark in mammals (Li and Zhang, 2014; Saksouk *et al*., 2015). Recent studies suggest that DNA methylation changes play a crucial role in the process of pain chronification (Denk and McMahon, 2012; Liang *et al*., 2015; Massara *et al*., 2016; Penas and Navarro, 2018). However, we are still far from understanding how DNA

methylation contributes to pain chronification and, specifically, which might be the role played by the crosstalk between miRNAs and this epigenetic mark in the neuropathic pain setting. In this regard, it has been reported that deregulation of miR-30c-5p, under several pathological conditions, brings about changes in the expression and function of DNMTs with pathophysiological implications (Gambacciani *et al*., 2013; Han *et al*., 2017). In addition, looking up in the online software based on bioinformatic algorithms (TargetScan4.0, miRanda and miRbase), we found that the "*de novo"* DNMT3b is consistently predicted as a target for miR-30c-5p.

Therefore, our next objective was to assess the occurrence of changes in the global levels of DNA methylation and in the distribution pattern of methylated DNA after SNI in pain related structures, the associated changes in DNMTs and the consequences of miR-30c-5p-gain and -loss of function.

5'-methylcitosine immunofluorescence constitutes a good surrogate of global DNA methylation that mainly occurs at the 5-carbon position of the pyrimidine ring of cytosine at the CpG sites (Jin *et al*., 2016). We carried out the immunofluorescence study in dissociates of primary sensory neurons of the sciatic nerve (L4–L6 DRGs) and second order nociceptive neurons of the lumbar SDH from sham-rats, and also in SNI-rats suffering from allodynia.

Our results evidence, for the first time, a robust increase in the methylated form of cytosine in the DNA of neurons in both nociception-related structures from SNI-animals, as reflected by the significant increase in the average fluorescence intensity and in the percentage of the nuclear area immunolabeled for 5'-methylcitosine.

In SNI-rats, 5'-methylcitosine was distributed through all the nucleoplasm except in DNA lacking areas, such as the speckles. Interestingly, accumulations of 5'-methylcitosine, especially in the perinucleolar region, were detected in sensitive neurons of the SDH. To our knowledge, this is the first study showing DNA-hypermethylation induced by SNI in neurons of the somatosensory nervous system, in association with neuropathic pain development.

Three major DNMTs, namely DNMT1, DNMT3a and DNMT3b, have been identified in mammals (Guz *et al*., 2010). DNMT1 is considered the primary

maintenance enzyme that plays a key role in keeping the pre-existing methylation marks in the newly synthesized DNA strands. The two "*de novo*" DNMT3a and DNMT3b methylate previously unmethylated CpG sequences in DNA (Jeltsch, 2006; Siedlecki and Zielenkiewicz, 2006). We assessed by qPCR and western blot the changes in the expression levels of DNMT3a, DNMT3b and DNMT1 in SDH and DRGs in our SNI model of neuropathic pain.

These findings indicate that the expression of DNMT3b was dramatically increased in the DRG and SDH neurons both 5 and 10 days after SNI. DNMT3a was also upregulated in the DRG 10 days after SNI. DNMT1 expression remained unchanged in both neural structures at any time after SNI. These results suggest that the hypermethylation of the DNA observed in DRG and SDH neurons after SNI might be mediated primarily through DNMT3b and, to a lesser extent, DNMT3a. On the other hand, the involvement of DNMT1 was not evidenced.

Our findings are consistent with previous published data showing DNMT3a and DNMT3b overexpression in neurons of the DRG and/or SDH using several models of sciatic nerve injury either in mice or rats (Pollema-Mays *et al*., 2014; Wang *et al*., 2016; Garriga *et al*., 2017; Sun *et al*., 2017; Zhao *et al*., 2017), and in inflammatory pain models (Tochiki *et al*., 2012). In contrast to our results, Zhao *et al* (2017) did not find differences in the expression of DNMT3b in the DRG of rats subjected to L5 spinal nerve ligation (SNL). This inconsistency could be accounted for by differences either in the timing of the determination or in the experimental models, being the spared SNI used in our study more harmful than SNL.

Overall, these results agree with our initial hypothesis that epigenetic modifications of chromatin can stabilize aberrant gene expressions believed to trigger/perpetuate neuropathic pain. Since DNA methylation is reversible and can be modulated by chemical agents, our results further highlight the *de novo* hypermethylation as an important epigenetic mark for the development of novel drugs to relief pain, and as an unexplored mechanism already involved in the analgesic effect of old drugs (i.e., opioids, NSAIDs, antidepressants, etc.) (Niederberger *et al*., 2017). Inhibition of *de novo* DNMT3b and/or DNMT3a might emerge as an effective strategy to reverse neuropathic pain through recovering the expression of pain-suppressor genes

that were silenced by methylation. In addition, an early intervention to reverse epigenetic alterations prompted by an acute nerve damage might prevent the progression to a chronic pain state.

Accumulating evidence over the past decade highlights the importance of miRNAs as key regulators of important physiological processes (Pradillo *et al*., 2018; Siddiqui *et al*., 2018; Vacante *et al*., 2018). Dysregulation of miRNA expression has been inferred in numerous diseases including neuropathic pain (Imai *et al*., 2011; Kusuda *et al*., 2011; *Genda et al*., 2013; Bali *et al*., 2014; Chang *et al*., 2017; Peng *et al*., 2017; Tramullas *et al*., 2018).

The canonical and best-known function of miRNAs is to negatively modulate gene expression post-transcriptionally. However, the crosstalk between miRNAs and other epigenetic marks depict a more complex layer of gene regulation (Bianchi *et al*., 2017); for example: a) the expression of miRNAs can be regulated by multiple epigenetic mechanisms (Saito *et al*., 2006); b) miRNAs can repress the expression of epigenetic factors (Miller *et al*., 2012; Xu *et al*., 2017); and c) miRNAs and epigenetic factors can cooperate to modulate common targets (Bao *et al*., 2004). These features highlight the important and complex roles played by miRNAs in the epigenetic control of gene expression. The characterization of the interactions between miRNAs and other epigenetic factors and the discovery coordinated regulatory networks, will facilitate the development of novel combined approaches to prevent and/or treat neuropathic pain.

As stated above, our experiments of miR-30c-5p modulation *in vivo* support a key role for miR-30c-5p in neuropathic pain development. Our previous results showing a long-lasting (two months) antiallodynic effect induced in SNI rats by knocking-down miR-30c-5p with a specific inhibitor (Tramullas *et al*., 2018), directed our interest towards assessing whether a possible crosstalk between miR-30c-5p and DNMTs could underlie such prolonged analgesia.

Based on these premises, we first determined, in the context of neuropathic pain, the influence of miR-30c-5p modulation on DNA methylation in isolated DRG and SDH neurons and on the expression of DNMTs in lysates of these tissues.

SNI rats treated with miR-30c-5p inhibitor, which were free of pain, exhibited even much higher DNA methylation levels than the neuropathic SNI-untreated-rats. DNA hypermethylation in DRG and SDH isolated neurons was paralleled by an intense up-regulation of the *de novo* DNMT3a and DNMT3b in lysates of both tissues, which could be related to new methylation patterns induced by the anti-miR in neurons and, probably, in non-neuronal cells. DNMT1 was also upregulated in both tissue lysates. This which might reflect an enhanced activity of DNA methylation maintenance either in non-neuronal dividing cells after DNA replication or in neuronal cells during DNA repair although the involvement of DNMT1 also in the *de novo* methylation of neuronal and non-neuronal cells is likewise plausible (Ren *et al*., 2018; Zhao *et al*., 2018).

Therefore, we suggest that miR-30c-5p loss-of-function pharmacologically induced after SNI results in analgesia by a mechanism involving DNA hypermethylation, through the three major DNMTs, and subsequent transcriptional repression of genes promoting neuropathic pain.

SNI-rats treated with miR-30c-5p mimic, whose allodynia at day 5 after SNI was intensified, presented methylation levels of the DNA significantly lower than those observed in untreated SNI-rats. It is conceivable that miR-30c-5p gain-of-function might reduce, either directly or indirectly, the methylation state of proallodynic genes, resulting in neuropathic pain potentiation.

We further analyzed whether the transcripts coding *de novo* DNMTs constitute direct targets of miR-30c-5p, using the luciferase reporter assay, a very reliable method to determine the capability of a miRNA to decrease luciferase activity in cells when it binds to its mRNA target (Thomson *et al*., 2011). We checked not only the interaction between miR-30c-5p and DNMT3b, which is predicted by online software, but also with DNMT3a, as suggested by our *in vivo* results and others (Gambacciani *et al*., 2014).

Our results showed a clear reduction in the luciferase activity when cells were co-transfected with miR-30c-5p mimic and the plasmids containing the 3'UTR regions of either DNMT3a or DNMT3b. Therefore, our *in vitro* results indicate that both DNMT3a and DNMT3b are direct targets of miR-30c-5p, confirming the prediction of online software regarding DNMT3b and adding DNMT3a to

the list of validated miR-30c-5p targets, as suggested by the experimental data (present results and Gambacciani *et al*., 2014).

If DNMT3a and DNMT3b were targets of miR-30c-5p their down-regulation should be expected under conditions of miR-30c-5p overexpression. In turn, miR-30c-5p loss-of-function would result in DNMT3a and DNMT3b up-regulation. Accordingly, in DRG from SNI-rats treated with miR-30c-5p mimic, the expression of both DNMT3a and DNMT3b was significantly lower than in untreated SNI rats, while the treatment miR-30c-5p inhibitor resulted in upregulation of DNMT3a and DNMT3b.

In contrast, the concurrent upregulation of miR-30c-5p (Tramullas *et al*., 2018) and *de novo* DNMTs in untreated SNI-rats did not fit into the canonical relationship between a miRNA and its target mRNA. Indeed, in such a complex pathophysiological processes as neuropathic pain, alteration of gene expression involves much more intricate interactions between players. In this way, under the neuropathic pain condition, numerous miRNAs, beside miR-30c-5p, could be contributing to regulate the expression of DNMTs and, in turn, DNMTs could be regulating the transcription of hundreds of miRNAs.

The relationship between neuropathic pain development and methylation changes in specific genes induced by nerve injury remains largely unknown. In this regard, it has been previously reported that DNMT3a overexpression in the DRG and/or SDH after sciatic nerve injury can contribute to neuropathic pain development by elevating DNA methylation in the promoter of genes encoding opioid receptors mu and kappa (Oprm1, Oprk1) and voltage-gated potassium channels (Kcna2) (Zhou *et al*., 2014; Sun *et al*., 2017; Zhao *et al.,* 2017; Shao *et al*., 2017). On the other hand, Jiang *et al.* (2017) reported downregulation of DNMT3b in the spinal cord after SNL, which may cause demethylation of the promoter regions of the chemokine receptor CXCR3 and the G-protein-coupled receptor 151 (GPR151) (Jiang *et al*., 2018). The subsequent spinal overexpression of these elements may contribute to neuropathic pain by facilitating central sensitization.

Herein, we assessed whether DNMT3a and DNMT3b regulation by miR-30c-5p may contribute to a differential DNA methylation profile in the promoter regions of pain-related genes that could explain the long-lasting antyallodynic effects observed after inhibition of miR-30c-5p.

In this regard, our first object of study was Nfyc, the hosting gene of miR-30c-5p. MiR-30c-5p is an intragenic intronic miRNA located within its host gene and transcribed under the control of the same promotor (UCSC, Genomics institute). In humans, nearly half of the known miRNAs are encoded within the introns of protein-coding genes. However, it is not clear whether intronic miRNAs are transcriptionally linked to their host genes or are transcribed independently (Steiman-Shimony *et al*., 2018).

Herein, we observed that Nfyc mRNA and miR-30c-5p expressions featured parallel changes under the different experimental conditions of our study in a positive linear relationship. We also show that the treatment with miR-30c-5p inhibitor reduced the expression values of Nfyc in SNI rats, while miR-30c-5p mimic tended to increase them. These results suggest that miR-30c-5p can regulate, either directly or indirectly, the expression of its hosting gene in the SDH and DRG.

If Nfyc were directly targeted by miR-30c-5p, a feature which is not predicted by the online informatics algorithms, the posttranscriptional regulation would have taken place in the opposite direction than the observed here (i.e. miR-30c-5p upregulation would induce Nfyc downregulation). Given that our results support DNMT3a and DNMT3b as miR-30c-5p targets, we hypothesize an indirect transcriptional regulation of Nfyc through DNA methylation. Accordingly with this idea, the methylation sensitive qPCR analysis indicated that the CpG sites in the promoter region of Nfyc were hypermethylated in SNI rats treated with miR-30c-5p inhibitor. Under the inhibition of miR-30c-5p, the *de novo* DNMT3s would recover their normal expression, allowing them to methylate the promoter region of Nfyc. The resultant transcriptional repression of Nfyc and, subsequently, of miR-30c-5p in nociception-related areas might contribute to the long-lasting anti-allodynic effect produced by treating SNI rats with miR-30c-5p inhibitor.

In this regard, there are several examples in the literature which report autoregulatory mechanisms of intronic miRNA on the expression and functions of their host genes, either through positive or negative feedback loops (Megraw *et al*. 2009; Dluzen *et al*., 2014. Steiman-Shimony *et al*., 2018). It should be bore in mind that, however, that the crosstalk between miR-30c-5p and its hosting gene could take place through many other

different mechanisms, including other miRNAs, depending on tissues and conditions.

The TGF-β is a family of pleiotropic, contextually acting cytokines (Massagué, 2012) that plays important roles in nociceptive processing (Lantero *et al*., 2012). In particular, many evidences obtained from animal models support that TGF-β1 prevents the neuronal plasticity underlying pain hypersensitization and promotes the activation of endogenous pain inhibitory pathways (Echeverry *et al.*, 2009; Tramullas *et al.*, 2010, 2018; Lantero *et al.*, 2012, 2014; Chen *et al.*, 2015). Our studies also provide evidence that TGF-β1 is a target of miR-30c-5p in the SDH of rodents under neuropathic pain conditions. Moreover, downregulation of TGF-β1 after SNI is a key mechanism underlying hypersensitization to nociceptive stimuli, whereas TGF-β1 gain-of-function results in allodynia relief or prevention (Tramullas *et al.*, 2018). Therefore, in this Doctoral Thesis, we further analyzed the regulatory networks between miR-30c-5p and TGF-β1 through epigenetic mechanisms.

Our present results show that the SDH from rats subjected to SNI featured aberrant CpG island DNA hyper-methylation in the promoter region of the gene coding TGFβ-1, in association with neuropathic pain. In contrast, pain-free SNI-rats treated with miR-30c-5p inhibitor showed promoter hypo-methylation. They therefore support that the methylation state of the TGFβ-1 promoter is regulated by miR-30c-related mechanisms with long-term consequences on pain perception.

The covalent post-translational modifications to histone proteins (methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation) constitute major epigenetic regulators of gene expression. Histone modifications usually take place in the lysine residues of the histone tails, and the changes on the chromatin state between euchromatin (transcriptional activation) and heterochromatin (transcriptional repression) depend on both the type of modification and the lysine that has been modified. Methylation of histone H3 at Lys9 or Lys27 (H3K9 or H3K27, respectively) or histone H4 at Lys20 (H4K20) usually leads to transcriptional repression, while methylation of H3K4, H3K36, and H3K79 generally produces transcriptional activation (Torres and Fujimori, 2015).

Recent research has shown that methylation of histone proteins and DNA have connected roles in the epigenetic control of gene expression. In mammals, DNA methylation in some genomic sites is dependent on histone methylation, and there is evidence of self-reinforcing loops between both epigenetic marks (Du *et al*., 2015). In this regard previous reports suggest that either the presence of H3K9me3 or the association of DNMT3a/b with the H3K9 methylation system might play a key role in targeting de novo DNA methylation at heterochromatic regions, although the precise molecular mechanism of these relationships remain poorly understood (Jackson *et al*., 2002; Lehnertz *et al*., 2003, Rose and Klose, 2014).

Our present findings indicate that, along with DNA hypermethylation, trimethylation of K9H3 was among the aberrant epigenetic mechanisms induced by SNI in the first (DRG) and second (SDH) order nociceptive neurons. Moreover, this repressive mark was under the control of miR-30c-5p to influence the pain-related behavior, as described above for DNA methylation. The direction in which such modulation took place resulted in drastic consequences on the nociception-related phenotype of the rats.

In the DRG neurons, increased expression levels of H3K9me3 were evidenced 5 days after SNI, when the severity of allodynia was still low. However, 10 days after SNI, when maximum allodynia was established, H3K9me3 recovered its baseline control levels. On the other hand, the treatment with miR-30c-5p mimic, which prevented H3K9me3 overexpression, potentiated the severity of the allodynia developed by SNI rats. Further, the dramatic overexpression of H3K9me3 produced by miR-30c-5p-loss-of-function did confer protection against allodynia development to SNI rats treated with the anti-miRNA.

The changes in H3K9me3, under the different experimental conditions, were paralleled by those of Suv39h1, the major methyltransferase that trimethylates the Lys9 of histone H3 (Rea *et al*., 2000).

Overall our findings suggest that SUV39H1/H3K9me3 overexpression might protect rats against neuropathic pain development, whereas the lower the expression of SUV39H1/H3K9me3, the higher the allodynia developed after SNI.

In full contradiction with our present results, Zhang *et al.* (2015) reported a direct causal relationship between SUV39H1 overexpression in SDH and DRG neurons and neuropathic pain development in rats subjected to the spinal nerve ligation model. Under their experimental conditions, blocking the activity or the expression of SUV39H1, using chaetocin or siRNA, protects against SNL-induced allodynia through a mechanism involving mu opioid receptors. However, several drawbacks of this study might be mentioned: first, the authors do not provide enough evidence of the success of their knockdown strategy, using intrathecal, DRG or intraspinal microinjections of SUV39H1 siRNA; second, chaetocin is a nonspecific inhibitor of histone lysine methyltransferases (Cherblanc *et al*., 2013); third, no evidence was provided of the changes in H3K9 methylation after the treatments with SUV39H1 siRNA or chaetocin.

Even though, our findings clearly support a protective role for H3K9 trimethylation against allodynia after knockdown miR-30c-5p with a specific inhibitor, more studies are needed to further delineate the role of this repressive epigenetic mark in neuropathic pain development.

Post-mitotic cells, particularly neurons, are very vulnerable to a variety of traumatic, metabolic, chemical and physical insults that could disrupt their homeostasis (McKinnon, 2009). Neuronal stress responses, on the one hand, encompass positive mechanisms in preserving or restoring neuronal function but, on the other hand, neuronal stress responses can also be deleterious, contributing to a broad range of neuropathologies (Farley and Watkins, 2018). Despite misconceptions that their regrowth is robust, the injured neurons projecting into the PNS retain only slow and incomplete regenerative capabilities (Doron-Mandel *et al*.,2015), and the affected part of the body is often subject to local sensory dysfunction or impaired motor function and to secondary problems such as neuropathic pain. Therefore, the almost permanent neurological consequences after severe injuries of peripheral nerves seriously affect the quality of life and patients' ability to work (Geuna *et al*., 2013).

DRG neurons are especially susceptible to damages due to their enormous metabolic activity, with high transcriptional and translational activity, which is required to maintain their cellular volume and bioelectric activity (Pena *et*

al., 2001). As an example of such vulnerability, it has been reported in rats that, several months after sciatic nerve section, approximately 35% of rat lumbar DRG neurons are lost by apoptosis (Welin *et al*., 2008).

Much research has been devoted to investigate the regenerative capability of peripherally injured neurons, including DRG sensitive neurons (Watson, 1974). Complete or partial injury to the sciatic nerve, one of the most widely used model in neuropathic pain studies (Colleoni *et al*., 2010; Austin *et al*., 2012), is often used in these studies. However, to the best of my knowledge, the possible relationship between the retrograde stress response to axotomy of DRG neurons and neuropathic pain development, prevention and/or resolution has never been addressed.

As an approach to this challenge, the next objective of this Thesis was to unravel whether the differential allodynia-related phenotype exhibited by rats treated with miR-30c-5p mimic or miR-30c-5p inhibitor could be related to differences in the neuronal retrograde response to SNI, paying particular attention to the protein synthesis machinery.

To this end, firstly, the Nissl bodies and the nucleolus of neurons dissociated from DRG were examined using as staining agent propidium iodide that, under our pH conditions, binds to RNA (Palanca *et al*., 2014). We also characterized the structural changes in the neuronal soma by electronic transmission microscopy.

Under control conditions, the Nissl bodies of non-injured DRG neurons are constituted by parallel arrays of cisterns of rough endoplasmic reticulum studded with ribosomes; rosettes of free polyribosomes and monoribosomes are found between the cisterns. Neural trauma (such as axon transection or crush), ischemia, toxics, metabolic diseases, and stress are well-known causes of the phenomenon termed central chromatolysis, a prominent neuropathological reactive response in the soma of damaged neurons (Torvik, 1976; Johnson and Sears 2013; Moon, 2018). Central chromatolysis involves the disruption, dispersal, and redistribution of the Nissl substance, leaving clear areas of empty cytoplasm (Torvik, 1976; Johnson and Sears 2013; Moon, 2018; Bradley *et al*., 2018).

In our study, almost 25% of the 3,000 neurons analyzed within L4, L5, and L6 DRG of SNI rats, at days 5 and 10 after SNI, exhibited all the hallmarks of central chromatolysis: loss and dissolution of Nissl substance in the center of the perikaryon, shifting of the remaining ribonucleoprotein complexes from the center towards the plasma membrane, nuclear elongation, folds in the nuclear envelope, and eccentric displacement of the nucleus towards the periphery of the cell.

Interestingly, in the group of SNI rats treated with miR-30c-5p mimic, the severity of chromatolysis increased significantly in parallel with the intensification of the allodynia developed at day 5 after SNI. Thus, almost half of the DRG neurons analyzed exhibited severe chromatolytic damage, accompanied of big oval shaped nuclei with numerous folds in the nuclear envelope, and shifted to an eccentric position in 13% of the neurons studied. In addition, an increase in the number and size of mitochondria also suggests alterations in the energy machinery.

By contrast, the treatment with miR-30c-5p inhibitor prevented the chromatolytic reaction of the injured DRG neurons and, in parallel, protected the rats against allodynia development 10 days after SNI. Thus, in the group of rats treated with miR-30c-5p inhibitor, the density and distribution of the Nissl substance exhibited a pattern similar to the sham-operated rats, and the presence of chromatolytic neurons was significantly reduced.

Overall, our results suggest that the severe disarray of the RER induced by SNI in DRG neurons is aggravated by miR-30c-5p-related mechanisms and might be related to neuropathic pain development.

In regenerating injured neurons, secondary compensatory anabolic processes usually exceed the initial catabolic processes of chromatolysis triggered by axotomy. Severely injured neurons can, however, remain chromatolytic and never again synthesize normal levels of protein, which results in atrophy or death (Moon, 2018). Therefore, our results could also suggests that targeting mir-30c-5p might result in an improvement of the survival rate of neurons following axotomy, although such hypothesis requires further analysis.

Among the possible mechanisms prompted by miR-30c-5p-gain-of-function to exacerbate chromatolysis could be the activation of catabolic pathways

mediated by ribonucleases and/or the impairment of anabolic processes such as rRNA synthesis for production of new ribosomes (Yang *et al*., 2018). However, given that miR-30c-5p mimic did not induce chromatolysis in non-injured sham neurons, we opted for the second hypothesis.

As a first approach to this issue, our next objective was to investigate whether RER dissolution associates to a functional disruption of the nucleolus, the cell compartment where the initial steps of ribosome biogenesis take place. The nucleolus maintains the structural and functional integrity of the RER by adapting the ribosome biogenesis to the cellular translation demands (Boulon *et al*., 2010).

The three main events for ribosome biogenesis (pre-rRNA transcription, processing, and ribosomal subunit assembly) are reflected in three distinct subnucleolar compartments named the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). It is generally accepted that pre-rRNA is transcribed from rDNA in the FC or at the border between the FC and DFC. FCs are enriched in components of the RNA Pol I machinery, such as UBF, whereas the DFC harbors pre-rRNA processing factors, such as the snoRNP proteins. Both the FC and the DFC are surrounded by the GC, where pre-ribosome subunit assembly takes place. The morphology and size of nucleoli are linked to nucleolar activity, which are inevitably altered under stress conditions, showing a variety of reorganization (Yang *et al*., 2018).

Immunofluorescence with UBF allowed us to define the number, structure and distribution of the FCs, parameters that are positively related to the transcriptional rate of the rDNA and, therefore, the functional state of the nucleolus (Berciano *et al*., 2007; Hernandez-Verdún *et al*., 2010).

Uninjured DRG neurons from sham rats exhibited the normal distribution pattern of UBF, with numerous small-sized UBF-positive spots that correspond with FC, which is characteristic of actively protein-synthesizing cells with high transcriptional activity (Berciano *et al*., 2007; Palanca *et al*., 2014). In contrast, the neurons from SNI rats, and especially those from SNI rats treated with miR-30c-5p mimic, showed severe structural alterations and loss of nucleolar structure, termed nucleolar segregation, which is characterized by the condensation and subsequent separation of the FC and GC, together with the formation of "nucleolar caps" (Boulon *et al*., 2010).

Severe segregation is associated with low rates of transcription and protein synthesis in other cell types (Raska *et al*., 2006; Smirnov *et al*., 2016) and, in neurons, is the typical manifestation of impaired RNA polymerase I activity induced by actinomycin D (Casafont *et al*., 2006; Lafita-Navarro *et al*., 2016). The nucleolar stress response is emerging as an important sensor of neuronal dysfunction in several neurodegenerative disorders (Baltanas *et al*., 2011; Parlato and Kreiner, 2013; García-Esparcia *et al*., 2015; Hernandez-Ortega *et al*., 2016).

Our results evidenced that the nucleolus is one of the cellular organelles affected by SNI, which is especially vulnerable to the harmful effect of miR-30c-5p overexpression. A deficient biogenesis of ribosomes, resultant from nucleolar dysfunction, is consistent with the severe chromatolytic alterations observed both in SNI rats and in SNI rats treated with miR-30c-5p mimic. Indeed, the contribution of other mechanisms, such as the activation of ribonucleases-mediated catabolic pathways cannot be completely excluded (Moon, 2018). However, it is noteworthy that, in conditions of severe chromatolysis under a variety of stressors (axotomy, treatment with proteasome inhibitors, amyotrophic lateral sclerosis), healthy nucleoli preserve their reticulated configuration and activate their transcriptional activity, as a compensatory response to promote functional recovery (Lafarga et al., 1991; Peters *et al*., 1991; Pena *et al*., 2001; Berciano *et al*., 2007; Palanca *et al*., 2014). Herein, administration of miR-30c-5p mimic to SNI rats, induced severe nucleolar segregation and, therefore, impairment of ribosomal biogenesis. In contrast, knockdown miR-30c-5p preserved the nucleolar integrity of injured DRG neurons and prevented chromatolysis following SNI.

These results strongly support that miR-30c-5p-gain-of-function might potentiate the nucleolar stress response induced by axotomy, turning the cell incompetent to compensate for the RER disruption and chromatolysis induced by axotomy.

The Cajal body (CB) is a multifunctional nuclear organelle, physically and functionally linked to the nucleolus. CBs have long been implicated in the assembly of snRNPs and snoRNPs. A classic example of CB-nucleolus cooperation are the snoRNPs required for rRNA processing within the DFC of

the nucleolus, which is assembled with the assistance of CBs (Lafarga *et al*., 2017; Massenet *et al*., 2017; Trinkle-Mulcahy and Sleeman, 2017).

Therefore, we further assessed the consequences of miR-30c-5p modulation following SNI on the CBs in dissociated DRG neurons immunostained for coilin, a molecular marker of CBs, and counterstained for RNA with propidium iodide.

We observed that, compared to the sham group, the proportion of neurons lacking CBs was significantly higher both in SNI rats and SNI rats treated with miR-30c-5p mimic. In contrast, there was a significant increase in the percentage of neurons showing two, three and even five CBs in DRG from SNI rats treated with miR-30c-5p inhibitor. Moreover, electron microscopy revealed that these neurons featured oversized CBs, some of them being as big as the nucleolus. Noteworthy, the changes observed in CBs did not affect their normal ultrastructure, but were rather quantitative.

It is well known that the size and number of CBs positively correlates with the transcriptional activity and the cellular processing demand of pre-mRNAs and pre-rRNA (Cioce and Lamond, 2005; Cioce *et al*.,2006; Jordan *et al*., 2007; Boulon *et al*., 2010; Machyna *et al*.,2013; Lafarga *et al*., 2017). Therefore, we suggest that the CBs were not disrupted by the SNI-related stress, and that the quantitative changes observed might be adaptive to accomplish the different nucleolar transcriptional activity of the neurons under each experimental condition. Thus, upon miR-30c-5p-gain-of-funcion, many neurons from SNI rats showed severely segregated and incompetent nucleoli, which secondarily resulted in lower number and size of CBs. In contrast, inhibition of miR-30c-5p would preserve the nucleolar structure and function following SNI, and the hypertrophied CBs would facilitate the maturation and transfer of the snoRNPs required by the nucleolus for processing pre-rRNAs to regenerate the ribosome subunits in damaged neurons.

In summary, our results suggest that miR-30c-5p-gain of function, following SNI, might potentiate the nucleolar stress reaction of DRG neurons to axotomy by inducing nucleolar segregation and secondary reduction of CBs size and number, which result in RER stress, dysfunction of ribosome biogenesis, and central chromatolysis. On the contrary, the DRG neurons

from SNI rats treated with miR-30c-5p inhibitor were protected against the global stress reaction of the nucleolus, CB and RER, which preserves the protein synthetic machinery.

It is becoming evident that miRNAs also have specific nuclear functions. Growing evidence supports nuclear regulatory roles for miRNAs on gene transcription, maturation of non-coding RNAs, alternative splicing, and maturation of rRNAs (Liao *et al*., 2010; Catalanotto *et al*., 2016). Given that miR-30c has been reported to be significantly concentrated in the nucleolus (Politz *et al*., 2007; Politz *et al*., 2009; Li *et al*., 2013; Bai *et al*., 2014), it is conceivable a nucleolar localized role for this miRNA in the response to stress.

The essential role of the nucleolus in coupling ribosome biogenesis to cell growth makes this structure a major target of signaling triggered by the cellular stress response (Boulon *et al*., 2010). Although the mechanisms controlling these stress pathways have been analyzed extensively, defining key targets susceptible of pharmacological manipulation to improve intrinsic neuroprotective mechanisms is still a challenge not achieved. Our study provides new insights on the role of miR-30c-5p in the stress-induced reorganization of the nucleolus and subsequent disruption of ribosome biogenesis, with a possible etiopathogenic link with neuropathic pain. Pharmacological knockdown of this new pathway might have relevant functional consequences on both nerve regeneration and neuropathic pain development.

Indeed, many issues of our study deserve further attention and will, undoubtedly, constitute some of the future lines of action of our laboratory. The following serve as examples: (i) verification of the causal relationship between the pro-allodynic effect of miR-30c-5p and nucleolar stress/chromatolysis (ii) definition of the miR-30c-5p downstream signal responsible for nucleolar stress and chromatolysis (iii) provision of preclinical proofs of concept for targeting mir-30c-5p, either directly or indirectly, to influence the regenerative capacity of DRG injured neurons and/or neuropathic pain development.

Conclusions

1. Neuropathic pain induced by sciatic nerve injury increases global DNA methylation levels in neurons of the dorsal root ganglia and spinal dorsal horn in rats.
2. Neuropathic pain induced by sciatic nerve injury increases the expression levels of DNMT3a and DNMT3b in dorsal root ganglia and of DNMT3b in the spinal dorsal horn in rats.
3. *In vivo* inhibition of miR-30c-5p after sciatic nerve injury increases the global DNA methylation levels in the dorsal root ganglia and spinal dorsal horn as well as the expression levels of DNMT3a, DNMT3b and DNMT1 in the dorsal root ganglia and expression levels of DNMT3b and DNMT1 in the spinal dorsal horn.
4. DNMT3a and DNMT3b are post-transcriptional targets of miR-30c-5p.
5. Indirect regulation of Nfyc gene by miR-30c-5p through DNA methylation in nociception-related areas might contribute to the long-lasting anti-allodynic effect of miR-30c-5p inhibitor after SNI.
6. The methylation state of the TGFB1 promoter is regulated by miR-30c-5p-related mechanism with long-term consequences on pain perception.
7. Neuropathic pain induced by sciatic nerve injury increases the levels of H3K9me3 at early stages in neurons of the dorsal root ganglia and spinal dorsal horn in rats.
8. *In vivo* inhibition of miR-30c-5p after sciatic nerve injury increases global methylation levels of H3K9me3 as well as the expression levels of Suv39h1 in the dorsal root ganglia and spinal dorsal horn.
9. Neuropathic pain induced by sciatic nerve injury induces central chromatolysis and nuclear eccentricity in dorsal root ganglia neurons.
10. miR-30c-5p mimic promotes the chromatolytic effect of sciatic nerve injury whereas miR-30c-5p inhibitor attenuates it.
11. Sciatic nerve injury induces segregation of the nucleolar components in DRGs neurons. miR-30c-5p gain of function potentiates the nucleolar

stress response induced by axotomy and miR-30c-5p knockdown preserves the nucleolar integrity of injured DRG neurons.

12. Neuropathic pain decreases the number of Cajal bodies in DRG neurons whereas inhibition of miR-30c-5p increases the number of Cajal bodies per neuron

Resumen en español

1. Introducción

El dolor, en condiciones fisiológicas, es una sensación desagradable que alerta al cuerpo de la presencia de daño tisular o enfermedad. La sensación dolorosa fisiológica es proporcional a la intensidad del estímulo y desencadena respuestas protectoras dirigidas a la defensa del organismo. Normalmente, el dolor desaparece tras la curación de la lesión que lo provocó. Sin embargo, en algunos individuos, y en determinadas situaciones de daño neural o inflamación, el dolor puede persistir mucho tiempo después de la curación de la lesión, debido al establecimiento de fenómenos de plasticidad patológica en el sistema nervioso (Basbaum *y cols*., 2009). Se califica como crónico a aquel dolor que se prolonga durante más de seis meses, a pesar de la curación de la causa que lo produjo y de carecer de función biológico-defensiva. El dolor crónico, al contrario que el dolor agudo, es considerado por la Organización Mundial de la Salud como una patología en sí mismo. La Sociedad Española del Dolor refiere que el 20% de la población española padece dolor crónico. El tiempo medio de evolución del dolor es de seis años y medio y, como consecuencia del mismo, el 30% de los pacientes se ven obligados a acogerse a la baja laboral.

El dolor neuropático es un tipo de dolor crónico, causado por la lesión o enfermedad del sistema nervioso somatosensorial (von Hehn *y cols*., 2012). Algunos individuos, tras la curación de una lesión nerviosa, desarrollan un cuadro de dolor persistente caracterizado por respuestas exageradas a los estímulos dolorosos (hiperalgesia), dolor en respuesta a estímulos inocuos (alodinia) y dolor espontáneo, que se mantienen durante meses e incluso años (Cerveró, 2009) y es altamente refractario a la terapia con analgésicos convencionales (Finerup *y cols*.,2015). El dolor neuropático es un proceso patológico muy prevalente, afectando a un 3-10% de la población general. Son causa frecuente de dolor neuropático la neuropatía secundaria a la isquemia crónica de las extremidades inferiores, el miembro fantasma tras la amputación de un miembro, los procesos tumorales, la neuropatía diabética, la neuralgia postherpética, el síndrome de dolor regional complejo, la fibromialgia, etc. (van Hecke *y cols.,*2014; Hsu y Cohen 2013).

La cronificación del dolor tras una lesión neural es consecuencia de un proceso de plasticidad patológica que se instaura en estructuras del sistema nervioso central y periférico responsables de la recepción, procesamiento y modulación de la sensibilidad nociceptiva (Basbaum *y cols*., 2009). El resultado final de estas adaptaciones patológicas es una hiperexcitabilidad neuronal de muy larga duración frente a estímulos nocivos o, incluso, inocuos. A pesar de que los mecanismos moleculares y celulares que contribuyen al fenómeno de sensibilización han sido exhaustivamente estudiados, desconocemos cuales son los elementos que determinan la persistencia del dolor tras la curación del daño neural y qué factores condicionan la susceptibilidad individual a padecer este tipo de patología.

El ganglio sensorial podrá constituir el primer nivel de alteración fisiopatológica de la modulación de la señalización aferente, en la medida en que permite la interacción entre diferentes tipos de información y parece ser un desencadenante del mecanismo de sensibilización central de las neuronas del asta dorsal de la médula espinal (Takeda *y colsl*., 2009).

Los mecanismos de regulación epigenética pueden incrementar o reducir la expresión de los genes sin alterar la secuencia primaria del DNA. El término epigenética hace referencia al conjunto de procesos químicos que modifican de forma duradera la actividad del DNA, sin alterar su secuencia. Un aspecto relevante es que, a diferencia de las mutaciones genéticas, las modificaciones epigenéticas pueden ser reversibles, bien en el contexto fisiológico, o mediante el uso de fármacos. En lo que al dolor se refiere, estudios recientes han puesto de manifiesto la existencia de cambios epigenéticos en neuronas y células gliales de la médula espinal y otras regiones superiores implicadas en el procesamiento nociceptivo en modelos experimentales de dolor crónico inflamatorio o neuropático (Tochiki *y cols* .,2012; Descalzi *y cols*.,2015; Liang *y cols*.,2015; Ligon *y cols*.,2016). Sistemas de neurotransmisión relacionados con la modulación del dolor, como es el sistema opioide endógeno (Muñoa *y cols*., 2015), canales iónicos implicados en la sensibilización nociceptiva (Laumet *y colsl*., 2015) también son susceptibles de regulación epigenética durante la cronificación del dolor. El conjunto de estudios permite proponer que la persistencia de la excitabilidad neuronal propia de los síndromes dolorosos crónicos podría estar relacionada con cambios epigenéticos dinámicos que modifican el fenotipo de

neuronas y células gliales. Los mecanismos de la maquinaria epigenética mejor estudiados son la metilación del DNA, la regulación de la estructura de la cromatina vía modificaciones de las histonas y los RNAs no codificantes, incluyendo los microRNAs (miRNAs). Los miRNAs son RNAs monocatenarios no-codificantes, de 19-25 nucleótidos, cuya función es la regulación post-transcripcional de la expresión génica, inhibiendo la traducción de mRNAs diana o promoviendo su degradación (Hobert, 2008). El resultado es una reducción de la expresión de proteínas diana. Resultados previos de nuestro grupo ponne de manifiesto un papel relevante para miR-30c-5p en el desarrollo de dolor neuropático, así como su potencial como biomarcador y diana terapéutica, tanto en el animal de experimentación como en pacientes (Tramullas *y cols*., 2018). En ratas sometidas a lesión del nervio ciático, la administración en la cisterna magna de miR-30c-5p mimic aceleró el desarrollo de dolor neuropático mientras que la administración de anti-miR-30c-5p retrasó la aparición de dolor neuropático. La relevancia de estos hallazgos pudimos trasladarla a la clínica en un grupo de pacientes con isquemia de las extremidades inferiores. Estos resultados sugieren fuertemente que: (i) miR-30c-5p juega un papel relevante en la plasticidad patológica del sistema nervioso en situaciones de dolor neuropático; (ii) terapias dirigidas a reducir la expresión de miR-30c-5p, o a incrementar la expresión de alguna de sus dianas, podrían tener valor para la prevención/curación del dolor neuropático; y (iii) los niveles circulantes de miR-30c podrían tener valor como biomarcadores accesibles.

Por otro lado, la enzima metiladora del DNA (DNMT3b) previamente relacionada con procesos de plasticidad neuronal inducida por actividad (memoria, adicción, long term potentiation, etc), (Fitzsimons, 2015) se encuentra entre los transcritos diana predichos para miR-30c-5p. Todo ello nos ha conducido a hipotetizar la existencia de cambios epigenéticos asociados a la persistencia del dolor tras la neuropatía, que podrían afectar tanto a las células neurales como a células circulantes de la sangre.

2. Objetivos

De acuerdo a todo lo expuesto anteriormente, proponemos los siguientes objetivos específicos:

1. Definir los cambios en el patrón de metilación global del DNA inducida por la lesión del nervio ciático en las neuronas de los ganglios dorsales (DRG) y el asta dorsal de la médula espinal (SDH), y las consecuencias de la ganancia y pérdida de función de miR-30c-5p.

2. Determinar la expresión de las DNA metiltransferasas (DNMT) en los DRG y SDH después de la lesión del nervio ciático y su modulación mediante la modulación de miR-30c-5p.

3. Validar *in vitro* DNMT3a y DNMT3b como dianas de miR-30c-5p en líneas celulares.

4. Evaluar la relación entre el estado de metilación de los genes relevantes en la nocicepción en el asta dorsal de la médula espinal y la intensidad del dolor neuropático después de la lesión del nervio ciático.

5. Definir los cambios de H3K9me3 inducidos por la lesión del nervio ciático en las neuronas de los DRG y SDH, y las consecuencias de la modulación de miR-30c-5p.

6. Determinar los niveles de expresión de Suv39h1 en los DRG y SDH tras la lesión del nervio ciático y su modulación mediante la manipulación de miR-30c-5p.

7. Evaluar en las neuronas DRG las consecuencias morfológicas y ultraestructurales de la ganancia y pérdida de función de miR-30c-5p- después de una lesión del nervio ciático. Específicamente, nos centraremos en la maquinaria de síntesis de proteínas: los grumos de Nissl, el núcleo y su compañero funcional, el cuerpo de Cajal.

3. Material y métodos

- *Sujetos de estudio y modelo experimental de dolor neuropático*

Para la realización de la presente Tesis Doctoral se emplearon ratas macho Sprague Dawley de 8-12 semanas de edad (250-300g). Se realizó el modelo de Spared nerve injury (SNI) descrito por Decosterd y Woold (2000) para causar el desarrollo de dolor crónico. Para ello se localizó el nervio ciático de la pata izquierda a la altura de la trifurcación en sus tres ramas y se realizó una axotomia con sutura de las ramas tibial y peroneal, dejando la rama sural intacta. Se valoró el grado de alodinia e hiperalgesia mecánica desarrollado mediante el test de von Frey. Para ello, la zona de la superficie plantar inervada por la rama sural de los animales fue estimulada 5 veces con microfilamentos de fuerza creciente. Cuando el animal realizó un movimiento de retirada, lamido o sacudida se consideró una respuesta positiva. El test finalizó cuando se obtuvo el 100% de respuestas para cada uno de los microfilamentos empleados.

- *Tratamientos y grupos experimentales*

Para determinar los efectos de la modulación de miR-30c-5p *in vivo* las ratas fueron anestesiadas con isofluorano e inmovilizadas en un instrumento stereotaxico. Los animales recibieron un ciclo de 3 inyecciones intracisternales de miR-30c-5p mimic (100 ng/μl) en el momento de la cirugía y los días 2 y 4 post-cirugía y un ciclo de 3 inyecciones de miR-30c-5p inhibidor (100 ng/μl) el día de la cirugía y los días 4 y 7 post-cirugía. Las ratas se sacrificaron cuando las diferencias en el grado de alodinia mecánica fueron máximas (días 5 y 10 post-cirugía respectivamente).

- *Procesamiento de las muestras*

Las ratas fueron decapitadas bajo anestesia con isofluorano para la realización de las técnicas de biología molecular. Se extrajo la médula espinal mediante hidroextrusión, seleccionando posteriormente la zona lumbar. A su vez, se obtuvieron los ganglios dorsales L3, L4 y L5.

Para la realización de las técnicas de inmunofluorescencia y microscopia electrónica, las ratas fueron anestesiadas y perfundidas con paraformahaldeido o con 3% glutaraldehido en tampón fosfato 0.12M respectivamente.

- *Técnicas bioquímicas: qPCR, WB, ms-qPCR*

Se extrajo el RNA de las muestras mediante el protocolo de Trizol y se obtuvo el cDNA mediante retrotranscripción usando un kit comercial (Revert-aidTM Minus First Strand cDNA synthesis kit, Fermentas). Para la retrotrascripción de los miRNAs, se utilizó el kit de μRT-PCR (Revert-aid, Fermentas). El análisis de la expresión génica se llevó a cabo mediante PCR cuantitativa a tiempo real.
El análisis de la expresión proteica se llevó a cabo mediante western blot empleando los anticuerpos primarios: DNMT3a:1/1000, DNMT3b:1/500 y Lamb1 1/1000.
El porcentaje de metilación de los genes Nfyc y TGFb-1 se determinó mediante PCR cuantitativa sensible a metilación (ms-qPCR). El DNA genómico se aisló del asta dorsal de la médula espinal empleando el kit NucleoSpin® Tissue (MN). Las muestras fueron digeridas con enzimas sensibles a metilación y amplificadas con primers específicos.

- *Microscopia laser confocal y electrónica*

Para la realización de los estudios de inmunofluorescencia, se realizó la técnica de los disociados neuronales de los ganglios dorsales y del asta dorsal de la médula espinal. Para ello, una pequeña porción de los tejidos perfundidos fue depositada en un portaobjetos superfrost y fue percutida con la ayuda de una aguja histológica. Las muestras se congelaron en hielo seco durante 5 min y estabilizadas en etanol 96% durante 7 min a 4ºC. Para la inmunofluorescencia convencional, los disociados neuronales fueron permeabilizados con PBS-Triton X-100 0.5% durante 45 min e incubados con el anticuerpo primario durante toda la noche (5'-MeC: 1/100, UBF: 1/100, Coilin: 1/300, Lamb1:1/100, H3K9me3:1/10) o con la solución de contraste Ioduro de propidio durante 20 min.

Para los estudios de microscopía electrónica realizamos el protocolo descrito por Palay and Chan-Palay (1974).Los tejidos perfundidos con glutaraldehido 3% en tampón fosfato 0.12M fueron post-fijados en tetróxido de osmio durante 2h y deshidradatas con soluciones de Acetona de porcentaje creciente. Los tejidos fueron incluidos en araldita (Durcapan) y dejados solidificar a 65ºC durante 48h.

- *Cultivos celulares: Dual Luciferasa reporter assay*

La regulación post-transcripcional de DNMT3a y DNMT3b se determinó mediante el ensayo de luciferasa en células HeLa y SH-SY5Y. El plásmido que contenía la región 3'-UTR de DNMT3a o DNMT3b (25nM) fue cotransfectado con miR-30c-5p mimic (10nM). Se midió la actividad luciferasa, que fue normalizada al total de proteína. El experimento fue replicado tres veces.

4. Resultados y discusión

Durante los últimos años, nuestro grupo de investigación se ha centrado en determinar el papel de los mecanismos epigenéticos relacionados con miRNAs en relación con las adaptaciones neuronales persistentes que se instauran tras la lesión del nervio periférico, lo cual contribuye al desarrollo de dolor neuropático. Nuestros resultados previos (Tramullas *y cols*., 2018) demuestran que miR-30c-5p tiene un papel relevante en el desarrollo y mantenimiento del dolor neuropático en modelos animales de lesión nerviosa y en pacientes con isquemia crítica que sufren dolor neuropático. Este estudio abre nuevas oportunidades terapéuticas enfocadas a interferir en el establecimiento de dolor crónico patológico y apoya el valor de miR-30c-5p como biomarcador para el dolor neuropático (Tramullas *y cols*., 2018).

En la presente Tesis Doctoral, nuestros objetivos fueron analizar en mayor profundidad los mecanismos epigenéticos que se activas tras la lesión del nervio ciático (SNI) que desencadena dolor neuropático y revelar la participación de miR-30c-5p.

La metilación del DNA, la cual se asocia con la formación de heterocromatina y silenciamiento génico constituye una de las más importante marcas epigenéticas. Estudios recientes sugieren que cambios en la metilación del

DNA juegan un papel crucial en el proceso de cronificación del dolor (Denk y McMahon, 2012; Massara y cols., 2016; Penas y Navarro, 2018). Sin embargo, se necesitan más estudios que determinen el papel de la metilación del DNA en la cronificación del dolor y específicamente, el papel que juega el crosstalk entre los miRNAs y esta marca epigenética en el contexto de dolor. En relación a ello, la desregulación de miR-30c-5p en varias condiciones patológicas implica cambios en la expresión y función de DNA metil transferasas (DNMTs) con implicaciones patofisiológicas (Gambacciani y cols., 2013; Han y cols., 2017). Además, DNMT3b se encuentra entre las dianas predichas de miR-30c-5p.

Por ello, nuestro primero objetivo fue determinar los cambios en los niveles globales de metilación del DNA y su patrón de distribución en dos áreas relacionadas con el dolor; el asta dorsal de la médula espinal (SDH) y los ganglios dorsales (DRG), los cambios asociados en los niveles de DNMTs y las consecuencias de la ganancia y pérdida de función de miR-30c-5p.

El estudio de inmunofluorescencia de 5'-Metilcitosina (5'-MeC) en disociados neuronales de DRG y SDH muestra, por primera vez, un fuerte incremento en los niveles de metilación del DNA, reflejado en un aumento significativo tanto en la intensidad promedio de las áreas marcadas como el porcentaje de área marcada tras la lesión del nervio ciático. En ratas SNI, 5'-MeC fue detectada en todo el nucleoplasma, excepto en zonas carentes de DNA como los speckles. Además observamos acumulaciones de 5'-MeC especialmente en las regiones perinucleolares en neuronas sensitivas del asta dorsal de la medula espinal. Para nuestro conocimiento, este es el primer estudio que muestra una hipermetilación global del DNA inducida por SNI en neuronas del sistema somatosensorial en relación con el desarrollo de dolor neuropático.

A continuación determinamos por qPCR y western blot cambios en los niveles de expresión de las tres principales DNMTs; DNMT3a, DNMT3b y DNMT1 en SDH y DRG en nuestro modelo de dolor neuropático. Nuestros resultados indican que la expresión de DNMT3b incrementó significativamente en DRG y SDH tras 5 y 10 días de la lesión del nervio ciático. DNMT3a también incremento tras 10 días de la lesión en los DRGs mientras que la expresión de DNMT1 no cambió. Nuestros resultados son consistentes con resultados

previos que demuestras un incremento en los niveles de DNMT3a y DNMT3 en neuronas del DRG y de la SDH en diversos modelos de lesión del nervio ciático en ratones y ratas (Pollema-Mays *y cols*., 2014; Wang *y cols*., 2016; Garriga *y cols*., 2017; Sun *y cols*., 2017; Zhao *y cols*., 2017). Estos resultados apoyan nuestra hipótesis inicial que postula que las modificaciones epigenéticas de la cromatina pueden estabilizar la expresión génica aberrante que subyace la cronificación del dolor neuropático.

Como descrito previamente, nuestros experimentos demuestran que la modulación *in vivo* de miR-30c-5p juega un papel fundamental en el desarrollo de dolor neuropático. Nuestros resultados previos muestrando el efecto anti-alodínico inducido en ratas SNI tras la administración de miR-30c-5p inhibidor (Tramullas *y cols*., 2018) dirigieron nuestro interés en determinar si un posible crosstalk entre miR-30c-5p y las DNMTs podría subyacer dicha analgesia.

En base a nuestras premisas, determinamos en primer lugar en el contexto de dolor neuropático la influencia de la modulación de miR-30c-5p en la metilación del DNA en neuronas aisladas de los DRGs y SDH, así como en la expresión de las DNMTs en lisados de estos tejidos. Las ratas SNI tratadas con miR-30c-5p inhibidor, las cuales están libres de dolor, presentaron unos niveles de metilación del DNA mucho mayores que las ratas SNI no tratadas. La hipermetilación del DNA en DRGs y SDH en neuronas aisladas se correlacionó con un intenso incremento en DNMT3a y DNMT3b en lisados de ambos tejidos, lo cual se puede relacionar con nuevos patrones de metilación inducidos por la inhibición de miR-30c-5p en neuronas y probablemente en células no neuronales. DNMT1 también incremento significativamente en ambos tejidos, lo cual podría reflejar una mayor actividad en el mantenimiento de la metilación del DNA. Por lo tanto, sugerimos que la perdida de función de miR-30c-5p tras la lesión del nervio ciático desencadena analgesia a través de un mecanismo mediado por la hipermetilación del DNA, a través de las tres principales DNMTs y una subsecuente represión transcripcional de genes que promueven el dolor neuropático.

Las ratas SNI tratadas con miR-30c-5p mimic, cuya alodinia a día 5 tras la lesión se intensificó, mostraron niveles de metilación del DNA significativamente menores que los observados en ratas SNI no tratadas.

Además, analizamos si DNMT3a y DNMT3b son dianas directas de miR-30c-5p, empleando el ensayo de luciferasa. Nuestros resultados muestras una clara reducción en la actividad luciferasa cuando las células fueron cotransfectadas con miR-30c-5p mimic y los plásmidos conteniendo la región 3'UTR de DNMT3a o DNMT3b. Por lo tanto, nuestros resultados indican que ambas *de novo* DNMTs son dianas directas de miR-30c-5p. Si DNMT3a and DNMT3b fueran dianas de miR-30c-5p, se debería esperar un incremento en sus niveles bajo condiciones de inhibición de miR-30c-5p, como bien muestran nuestros resultados.

Tras ello, quisimos determinar si la regulación de DNMT3a y DNMT3b por miR-30c-5p podría contribuir a un patrón de metilación del DNA diferencial en las regiones promotoras de genes relacionados con el dolor que pudieran explicar los efectos antialodínicos duraderos observados tras la inhibición de miR-30c-5p. Para ello, estudiamos en primer lugar Nfyc, el gen que contiene la secuencia de miR-30c-5p. Observamos que las expresiones de Nfyc y miR-30c-5p mostraron cambios paralelos en las diferentes condiciones experimentales de nuestro estudio de una manera lineal positiva. El tratamiento con miR-30c-5p inhibidor redujo los niveles de expresión de Nfyc mientras que miR-30c-5p mimic mostró una tendencia a incrementarlos. Estos resultados sugieren que miR-30c-5p puede regular directa o indirectamente la expresión de su propio gen en los DRG y SDH. Dado que nuestros resultados demuestran que DNMT3a y DNMT3b son dianas post-transcripcionales de miR-30c-5p, hipotetizamos una regulación transcripcional indirecta de Nfyc a través de la metilación del DNA. La técnica de la PCR sensible a metilación (ms-qPCR) indicó que las islas CpG en la región promotora de Nfyc se encontraban hipermetiladas en ratas SNI tratadas con miR-30c-5p inhibidor. Tras la inhibición de miR-30c-5p, las dos *de novo* DNMTs (DNMT3a y DNMT3b) recuperaran su expresión normal, pudiendo metilar el promotor de Nfyc. La represión transcripcional resultante de Nfyc podría contribuir a los efectos antialodínicos que se producen en ratas SNI tratadas con miR-30c-5p inhibidor.

Nuestros estudios previos también proporcionan evidencias de que TGF- β1 es diana de miR-30c-5p y que la disminución de TGFB tras SNI es un mecanimso clave subyacente a la hipersensibilización a los estimulos nocivos mientras que la ganancia de función de TGF-b1 resulta en una prevención de la alodinia (Tramullas *y cols*., 2018). Los resultados obtenidos en la presente Tesis doctoral muestran que en SDH de ratas SNI mostraron una hipermetilacion aberrante del DNA en la región promotora de TGFB1. Por el contrario las ratas SIN tratadas con miR-30c-5p inhibidor mostraron una hipometilación del promotor. Por lo tanto, nuestros resultados apoyan que el estado de metilación del promotor de TGFB1 es regulado a través de mecanismos relacionados con miR-30c-5p con consecuencias a largo plazo en la percepción del dolor.

Por otra parte, nuestros resultados también demuestras que, junto con la hipermetilación del DNA, la trimetilacion de H3K9 se encuentra entre los mecanismos epigeneticos aberrantes inducidos por la lesión del nervio ciático en la primera (DRG) y segunda (SDH) neurona nociceptiva. Además, esta marca de represión génica también se encuentra bajo el control de miR-30c-5p. En neuronas de los DRGs, se evidenció un incremento en la expresión de H3K9me3 tras 5 días de la lesión nerviosa. Sin embargo, tras 10 días de la lesión, cuando se observan los máximos niveles de alodinia, se recuperaron los niveles normales de H3K9me3. Además, el tratamiento con miR-30c-5p inhibidor condujo a un aumento drástico en los niveles de H3K9me3, lo cual parece conferir protección frente al desarrollo de alodinia en ratas SIN tratadas con miR-30c-5p inhibidor. Los cambios en H3K9me3 en las diferentes condiciones experimentales se correlacionaron con cambios en Suv39h1, la principal histona metil transferasa que trimetila la lisina 9 de la histona H (Rea *y cols*., 2000).En conjunto, nuestros resultados sugieren que la sobreexpresión de Suv39h1/H3K9me3 protege a las ratas del desarrollo de dolor neuropático, mientras que niveles bajos de Suv39h1/H3K9me3 se relacionan con un mayor grado de alodinia.

Las células post-mitóticas, particularmente las neuronas son muy vulnerables a una gran variedad de eventos traumáticos, metabólicos, químicos y físicos que pueden alterar su homeostasis (McKinnon, 2009). Las neuronas DRGs son especialmente susceptibles al daño debido a su gran actividad metabólica

y su alta actividad transcripcional y traduccional. El siguiente objetivo de esta Tesis fue determinar si el fenotipo alodínico diferencial mostrados por las ratas tratadas con miR-30c-5p mimic o miR-30c-5p inhibidor podría relacionarse con diferencias en la respuesta neuronal retrógrada a la lesión del nervio ciático, mostrando particular atención en la maquinaria de síntesis protéica. En nuestro estudio, casi un 25% de las 3,000 neuronas analizadas en los ganglios L4,L5 y L6 de ratas SNI tras 5 y 10 días de la lesión, mostraron todas las características de la cromatolisis central: perdida y disolución de los grumos de Nissl, elongación nuclear y desplazamiento del núcleo hacia la periferia de la celula. De manera interesante, el grupo de ratas SIN tratadas con miR-30c-5p mimic la severidad de la cromatolisis aumento significativamente en paralelo con la intensificación de la alodinia desarrollada tras 5 dias de la lesión. Ello fue acompañado con excentricidad nuclear (13%). Por el contrario, el tratamiento con miR-30c-5p inhibidor previno la reacción cromatolítica en neuronas DRGs. Por lo tanto, en el grupo de ratas tratadas con miR-30c-5p inhibidor la densidad y distribución de los grumos de Nissl mostró un patrón similar a las ratas sham y la presencia de neuronas con cromatolisis se redujo significativamente. Uno de los posibles mecanismos desencadenados por la ganancia de función de miR-30c-5p para incrmentar la cromatolisis podría implicar la disfunción de la síntesis de rRNA para la producción de nuevos ribosomas. Por ello, nuestro siguiente objetivo fue investigar si la disolución de los grumos de Nissl se asociaba con cambios funcionales del nucleolo, el orgánulo donde los pasos iniciales para la síntesis de los ribosomas tienen lugar. La inmunofluorescencia para marcar el factor de transcripción UBF (localizado en los centros fibrilares del nucleolo) reveló que en neuronas de DRGs de ratas sham, UBS mostró el patrón de distribución normal con numerosos puntos UBS-positivos distribuidos a lo largo de todo el nucleoplasma, lo cual es característico de células con una actividad transcripcional alta y una buena maquinaria de síntesis de proteínas. Por el contrario, las neuronas de ratas SNI y especialmente de aquellas tratadas con miR-30c-5p mimic mostraron alteraciones nucleolares severas y pérdida de la estructura nucleolar (segregación del componente fibrilar denso). Esta segregación ha sido asociada con bajas tasas de transcripción y de síntesis de proteínas tanto en neuronas (Casafont *y cols*., 2006; Lafita-Navarro *y cols*., 2016) como en otros tipos celulates (Raska y

cols., 2006; Smirnov *y cols*., 2016). Nuestros resultados evidencian que el nucleolo es uno de los orgánulos celulares afectados por la lesión del nervio ciático, el cual es especialmente vulnerable al efecto dañino de la sobreexpresión de miR-30c-5p. Una biogénesis ribosomal deficiente resultado de la disfunción ribosomal es consistente con las alteraciones cromatoliticas observadas en ratas SNI y ratas SIN tratadas con miR-30c-5p mimic.

Por último, investigamos las consecuencias de la modulación de miR-30c-5p tras la lesión del nervio ciático sobre un orgánulo nuclear multifuncional que está físicamente y funcionalmente relacionado con el nucleolo, el cuerpo de Cajal (CB). El CB está implicado en la biogénesis de las snRNPs y snoRNPs, necesarias para el procesamientode los mRNAs y rRNAs respectivamente. Observamos que en comparación con las ratas sham, el porcentaje de neuronas que carecían de CBs fue significativamente mayor en ratas SNI y ratas SNI tratadas con miR-30c-5p mimic. Por el contrario, observamos un incremento significativo en el porcentaje de neuronas mostrando dos, tres e incluso cinco CBs en DRGs de ratas SNI tratadas con miR-30c-5p inhibidor. El número y tamaño de los CBs se corerlaciona positivamente con la actividad transcripcional celular (Cioce y Lamond, 2005; Cioce *y cols*.2006; Lafarga y cols., 2017) por lo que sugerimos que los cambios observados en el número de CBs pueden ser adaptativos para compensar la diferente actividad transcripcional nucleolar de las neuronas bajo cada condición experimental.

Recientes estudios demuestran que los miRNAs también tienen funciones nucleares específicas. Además, dado que se ha demostrado que miR-30c se encuentra significativamente concentrado en el nucleolo (Politz *y cols*., 2007; Politz *y cols*., 2009; Li *y cols*., 2013; Bai y cols., 2014), es posible que miR-30c tenga un papel nucleolar en respuesta al estrés.

Muchos temas de nuestro estudio merecen mayor atención y, sin duda, constituirán algunas de las líneas de acción futuras de nuestro laboratorio. Los siguientes son algunos ejemplos: (i) verificación de la relación causal entre el efecto pro-alodínico de miR-30c-5p y el estrés nucleolar y cromatolisis (ii) definición de la señal downstream de miR-30c-5p responsable del estrés nucleolar.

5. Conclusiones

1. El dolor neuropático inducido por la lesión del nervio ciático aumenta los niveles globales de metilación del DNA en neuronas de los ganglios dorsales (DRG) y del asta dorsal de la médula espinal (SDH).

2. El dolor neuropático inducido por la lesión del nervio ciático aumenta los niveles de expresión de DNMT3a y DNMT3b en los DRG y de DNMT3b en SDH.

3. La inhibición *in vivo* de miR-30c-5p tras la lesión del nervio ciático aumenta los niveles globales de metilación del DNA en neruonas de los DRG y SDH, así como los niveles de expresión de DNMT3a, DNMT3b y DNMT1 en DRG y de DNMT3b y DNMT1 en SDH.

4. DNMT3a y DNMT3b son dianas post-transcripcionales de miR-30c-5p.

5. La regulación indirecta del gen Nfyc por miR-30c-5p a través de la metilación del DNA en áreas relacionadas con la nocicepción podría contribuir al efecto antialodínico de la inhibición de miR-30c-5p tras SNI.

6. El estado de metilación del promotor TGF-β1 está regulado por un mecanismo relacionado con miR-30c-5p con consecuencias a largo plazo en la percepción del dolor.

7. El dolor neuropático inducido por la lesión del nervio ciático aumenta en las etapas iniciales los niveles de H3K9me3 en neuronas de los DRG y SDH.

8. La inhibición *in vivo* de miR-30c-5p tras la lesión del nervio ciático aumenta los niveles de H3K9me3, así como los niveles de expresión de Suv39h1 en DRG y SDH.

9. El dolor neuropático inducido por la lesión del nervio ciático induce cromatolisi central y excentricidad nuclear en las neuronas de los DRG.

10. miR-30c-5p mimic promueve el efecto cromatolítico de la lesión del nervio ciático, mientras que miR-30c-5p inhibidor lo atenúa.

11. La lesión del nervio ciático induce la segregación de los componentes nucleolares en las neuronas DRG. La ganancia de función de miR-30c-5p potencia la respuesta al estrés nucleolar inducida por la axotomía y la

reducción de miR-30c-5p preserva la integridad nucleolar de las neuronas DRG lesionadas.

12. El dolor neuropático disminuye el número de cuerpos de Cajal en las neuronas DRG, mientras que la inhibición de miR-30c-5p aumenta el número de cuerpos de Cajal por neurona

Bibliography

Adachi T, Nakanishi M, Otsuka Y, Nishimura K, Hirokawa G, Goto Y, Nonogi H, Iwai N. "Plasma microRNA 499 as a biomarker of acute myocardial infarction". Clinical Chemistry 56(7):1183-1185 (2010).

Adlakha YK, Saini N." Brain microRNAs and insights into biological functions and therapeutic potential of brain enriched miRNA-128". Mol Cancer 21; 13:33 (2014).

Afzal TA, Luong LA, Chen D, Zhang C, Yang F, Chen Q, An W, Wilkes E, Yashiro K, Cutillas PR, Zhang L, Xiao Q." NCK Associated Protein 1 Modulated by miRNA-214 Determines Vascular Smooth Muscle Cell Migration, Proliferation, and Neointima Hyperplasia". J Am Heart Assoc 7; 5(12) (2016).

Agostini M, Tucci P, Steinert JR, Shalom-Feuerstein R, Rouleau M, Aberdam D, Forsythe ID, Young KW, Ventura A, Concepción CP, Han YC, Candi E, Knight RA, Mak TW, Melino G. "MicroRNA-34a regulates neurite outgrowth, spinal morphology, and function". Proceedigs of the National Academy of Science U.S.A. 108:21099–21104 (2011).

Ahluwalia A, Yan P, Hurteau JA, Bigsby RM, Jung SH, Huang TH, Nephew KP." DNA methylation and ovarian cancer. I. Analysis of CpG island hypermethylation in human ovarian cancer using differential methylation hybridization". Gynecl Oncol 82(2):261-8 (2001).

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. " Molecular Biology of the Cell,4th edition". (2002).

Aldrich BT, Frankes EP, Kasuya J, Hammond DL, Kitamoto T. "Changes in expression of sensory organ specific microRNAs in rat dorsal root ganglia in association with mechanical hiper-sensitivity induced by spinal nerve ligation". Neuroscience 164(2):711-723 (2009).

Alevizos I, Illei GG. "MicroRNAs as biomarkers in rheumatic diseases". Nature Reviews Rheumatology 6(7):391-398 (2010).

Aliaga L, Baños JE, De Barutell C, Molet J and Rodríguez de la Serna A. Tratamiento del dolor. Teoría y práctica. 3ª edición Permanyer (2009).

Alsharafi W and Xiao B. "Dynamic Expression of MicroRNAs (183, 135a, 125b, 128, 30c and 27a) in the Rat Pilocarpine Model and Temporal Lobe Epilepsy Patients". CNS Neurol Disord Drug Targets". 14(8):1096-102 (2015).

Ambros V." The functions of animal microRNAs". Nature 16; 434:350-5 (2004).

Anderson D, Anderson , Chang C, Makarewich C, Nelson B, McAnally J, et al. . A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell 160, 595–606 (2015).

Andersen M, Trapani D, Ravn J, Sørensen JB, Andersen CB, Grauslund M, Santoni-Rugiu E." Methylation-associated Silencing of microRNA-126 and its Host Gene EGFL7 in Malignant Pleural Mesothelioma". Anticancer Res 35(11):6223-9 (2015).

Apkarian AV, Hashmi JA and Baliki MN. "Pain and the brain: specificity and plasticity of the brain in clinical chronic pain". Pain 152(3):S49-S64 (2011).

Arai M, Genda Y, Ishikawa M, Shunsuke T, Okabe T, Sakamoto A. "The miRNA and mRNA changes in rat hippocampi after chronic constriction injury". Pain Medicine 14(5):720-729 (2013).

Arifuzzaman S, Das A, Kim SH, Yoon T, Lee YS, Jung KH, Chai YG." Selective inhibition of EZH2 by a small molecule inhibitor regulates microglial gene expression essential for inflammation". Biochem Pharmacol 1; 137:61-80 (2017).

Auclair G, Guibert S, Bender A, Weber M." Ontogeny of CpG island methylation and specificity of DNMT3 methyltransferases during embryonic development in the mouse". Genome Biol 15(12):545 (2014).

Austin PJ and Moalem-Taylor G." The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines". J Neuroimmunol 15; 229 (1-2):26-50 (2010).

Austin PJ, Wu M, and Moalem-Taylor G. "Chronic Constriction of the Sciatic Nerve and Pain Hypersensitivity Testing in Rats". 61:3393 (2012).

Bai G, Wei D, Zou S, Ren K, Dubner R.” Inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia”. Mol Pain 7; 6:51 (2010).

Bai B, Lui H and Laiho M. “Small RNA expression and deep sequencing analyses of the nucleolus reveal the presence of nucleolus-associated microRNAs”. FEBS. 4. 441-449 (2014).

Bali KK, Hackenberg M, Lubin A, Kuner R and Devor M. “Sources of individual variability: miRNAs that predispose to neuropathic pain identified using genome-wide sequencing”. Mol. Pain 10:22(2014).

Baltanás FC,Casafont I,Weruaga E,Alonso JR,Berciano MT, Lafarga M.” Nucleolar disruption and cajal body disassembly are nuclear hallmarks of DNA damage-induced neurodegeneration in purkinje cells”. Brain Pathol 21(4):374-88 (2011).

Bansal A, Pinney SE.” DNA methylation and its role in the pathogenesis of diabetes”. Pediatr Diabetes 18(3):167-177 (2017).

Bao N, Lye KW and Barton MK.” MicroRNA binding sites in Arabidopsis class III HD-ZIP mRNAs are required for methylation of the template chromosome”. Dev Cell 7(5):653-62 (2004).

Baraniskin A, Kuhnhenn J, Schlegel U, Chan A, Deckert M, Gold R, Maghnouj A, Zöllner H, Reinacher-Schick A, Schmiegel W, Hahn SA and Schroers R.” Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system”. Blood; 117(11):3140-6(2011).

Baron R, Binder A, Wasner G. “Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment”. Lancet Neurol 9(8):807-19 (2010).

Bartel, DP. “MicroRNAs: genomics, biogenesis, mechanism, and function”. Cell 116(2):281–297 (2004).

Bartel DP. “MicroRNAs: target recognition and regulatory functions”. Cell 136(2):215-233 (2009).

Basbaum A, Bautista D, Scherrer G and Julius D. "Cellular and molecular mechanisms of Pain". Cell. 139, 267–284 (2009).

Baskerville S and Bartel DP." Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes". RNA 11(3):241-247 (2005).

Berciano MT, Novell M, Villagra NT, Casafont I, Bengoechea R, Val-Bernal JF and Lafarga M. "Cajal body number and nucleolar size correlate with the cell body mass in human sensory ganglia neurons". J Struct Biol 158(3):410-420 (2007).

Bermejo JL, Huang G, Manoochehri M, Mesa KG, Schick M, Silos RG, Ko YD, Brüning T, Brauch H, Lo WY, Hoheisel JD, Hamann U." Long intergenic noncoding RNA 299 methylation in peripheral blood is a biomarker for triple-negative breast cancer". Epigenomics (2018).

Bestor TH." The DNA methyltransferases of mammals". Hum Mol Genet 9; 16:2395-402 (2000).

BhattacharyyaSN, Habermacher R, Martine U, Closs EI, Filipowicz W." Relief of microRNA-mediated translational repression in human cells subjected to stress". Cell 16; 125(6):1111-24 (2006).

Bhaumik SR1, Smith E andShilatifard A." Covalent modifications of histones during development and disease pathogenesis". Nat Struct Mol Biol 14(11):1008-16 (2007).

Bianchi M, Renzini A,Adamo S and Moresi V. " Coordinated Actions of MicroRNAs with other Epigenetic Factors Regul ate Skeletal MuscleDevelopment and Adaptation". Int J Mol Sci 15; 18 (4) (2017).

Bird A." CpG-rich islands and the function of DNA methylation". Nature 15-21; 321 (1986).

Bird A. "DNA methylation patterns and epigenetic memory". Genes Dev 1; 16(1):6-21 (2002).

Bird A."Perceptions of epigenetics". Nature 24; 447(7143):396-8 (2007).

Bohnsack MT,Czaplinski K,Gorlich D.” Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs”. RNA 10(2):185-91 (2004).

Boisvert FM,van Koningsbruggen S, Navascués J,Lamond AI.” The multifunctional nucleolus”. Nat Rev Mol Cell Biol 8(7):574-85 (2007).

BoivinV,Deschamps-Francoeur G andScott MS.” Protein coding genes as hosts for noncoding RNA expression”. Samin Cell Dev Biol 75:3-12 (2017).

Bonica JJ. “Bonica´s Management of Pain”. Lippincott Williams y Wilkins. Eds. Scott Fishman, Jane Ballantyne, and James P. Rathmell (2001).

Bonin RP, Bories C and De Koninck Y. “A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments”. Molecular Pain 10:26 (2014).

BoulonS, Westman BJ, Hutten S, Boisvert FM and Lamond AI.” The nucleolus under stress”. Molecular Cell 39(6):912-924 (2010).

Bradley A, Bertrand L, Rao D, Hall D, Sharma A. Chapter 13, Brain. Boorman’s Pathology of the Rat (Second edition) 191-215 (2018).

Brandenburger T, Castoldi M, Brendel M, Grievink H, Schlösser L, Werdehausen R, Bauer I, Hermanns H. “Expression of spinal cord miRNAs in a rat model of chronic neuropathic pain”. Neuroscience Letters 506(2):281-286 (2012).

Brennecke J, Stark A, Russell RB, Cohen SM. “Principles of microRNA-target recognition”. PLoS Biology 3(3):e85 (2005).

Bridge G, Monteiro R, Henderson S, Emuss V, Lagos D, Georgopoulou D, Patient R, Boshoff C. “The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis”. Blood 120 (25):5063-72 (2012).

Buchheit T, Van de Ven T and Shaw A.” Epigenetics and the transition from acute to chronic pain”. Pain Med 13(11):1474-90 (2012).

Burger K,Mühl B,Harasim T,Rohrmoser M,Malamoussi A,Orban M,Kellner M,Gruber-Eber A,Kremmer E,Hölzel M,Eick D.” Chemotherapeutic

drugs inhibit ribosome biogenesis at various levels". J Biol Chem 16; 285(16):12416-25 (2010).

Burgos K, Metpally R, Courtright A, Rakela B, Beach T, Shill H, Adler C, Sabbagh M, Villa S, Tembe W, Craig D, Van Keuren-Jensen K." Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology". PLoS one 5; 9(5) (2014).

Bursać S, Brdovčak MC, Pfannkuchen M, Orsolić I, Golomb L, Zhu Y, Katz C, Daftuar L, Grabušić K, Vukelić I." Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress". Proc. Natl. Acad. Sci. USA 109:20467–20472 8 (2012).

Butz H, Rácz K, Hunyady L, Patócs A. "Crosstalk between TGF-β signaling and the microRNA machinery". Trends in Pharmacological Sciences 33(7):382-393 (2012).

Callie R. Merry, Megan E. Forrest, Jessica N. Sabers, Lydia Beard, Xing-Huang Gao, Maria Hatzoglou, Mark W. Jackson, Zhenghe Wang, Sanford D. Markowitz, Ahmad M. Khalil." DNMT1-associated long non-coding RNAs regulate global gene expression and DNA methylation in colon cancer". Hum Mol Genet 24(21):6240-6253(2015).

Calvo M, Dawes JM, Bennett DL. "The role of the immune system in the generation of neuropathic pain". Lancet Neurology 11(7):629- 642 (2012).

Campbell JN and Meyer RA. "Mechanisms of neuropathic pain". Neuron 52(1):77-92 (2006).

Capasso KE, Manners MT, Quershi RA, Tian Y, Gao R, Hu H, Barrett JE, Sacan A, Ajit SK." Effect of histone deacetylase inhibitor JNJ-26481585 in pain". J Mol Neurosci 55(3):570-8 (2015).

Catalanotto C, Cogoni C, Zardo G. "MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions". Int J Mol Sci (2016).

Casafont I, Navascués J, Pena E, Lafarga M, Berciano MT.” Nuclear organization and dynamics of transcription sites in rat sensory ganglia neurons detected by incorporation of 5'-fluorouridine into nascent RNA”. Neurosci 30; 140(2):453-62 (2006).

Ceccacci E, Minucci S.” Inhibition of histone deacetylases in cancer therapy: lessons from leukaemia”. Br J Cancer 15; 114(6):605-11 (2016).

Cerdá-Olmedo G, Mena-Durán AV, Monsalve V and Oltra E.” Identification of a microRNA signature for the diagnosis of fibromyalgia”. Plos One, 10 (3) (2015).

Cerveró F, Laird JM. “Understanding the signaling and transmission of visceral nociceptive events”. J Neurobiol.61.1: 45-54 (2004).

Cerveró F.” Pain: friend or foe? A Neurobiologic perspective: the 2008 Bonica Award Lecture”. Reg Anesth Pain Med 34(6):569-74 (2009).

Chang PY, Chen YJ, Huang WH, Yang TC, Lee YT, Chang SF, Lu SC and Chen CH.”
Aspirin protects human coronary artery endothelial cells against athero genic electronegative LDLvia an epigenetic mechanism:
a novel cytoprotective role of aspirin in acute myocardialinfarction”.
Cardiovas Res 1; 99 (1):137-45 (2013).

Chang HL, Wang HC, Chunag YT, Chou CW, Lin IL, Lai CS, Chang LL and Cheng KI. “miRNA expression change in dorsal root ganglia after peripheral nerve injury. *J. Mol. Neurosci.* 61 169–177 (2017).

Chen K and Rajewsky N.” Natural selection on human microRNA binding sites inferred from SNP data”.Nat Genet 38(12):1452-6 (2006).

Chen T and Li E. Establishment and maintenance of DNA methylation patterns in mammals. Curr. Top. Microbiol. Immunol 301:179–201 (2006).

Chen HP, Zhou W, Kang LM, Yan H, Zhang L, Xu BH, Cai WH.” Intrathecal miR-96 inhibits Nav1.3 expression and alleviates neuropathic pain in rat following chronic construction injury”. Neurochem Res 39(1):76-83 (2014).

Chen G, Park CK, Xie RG, Ji RR.” Intrathecal bone marrow stromal cells inhibit neuropathic pain via TGF-β secretion”. J Clin Invset 3; 125 (8):3226-40 (2015).

Chen H, Orozco LD, Wang J, Rau CD, Rubbi L, Ren S, Wang Y, Pellegrini M, Lusis AJ, Vondriska TM.” DNA Methylation Indicates Susceptibility to Isoproterenol-Induced Cardiac Pathology and Is Associated With Chromatin States”. Circ Res 4; 118(5):786-97 (2016).

Chen X, Xiao Y, Wei L, Wu Y, Lu J, Guo W, Huang S, Zhou M, Mo M, Li Z, Cen L, Li S, Yang C, Wu Z, Hu S, Pei Z, Yang X, Qu S, Xu P.” Association of DNMT3b gene variants with sporadic Parkinson's disease in a Chinese Hanpopulation”. J Gene Med 19(11); 360-365 (2017).

Chen J, Yu Y, Xue C, Chen X, Cui G, Li J, Li K, Ren Z, Sun R.” Low microRNA-139 expression associates with poor prognosis in patients with tumors: A meta-analysis”. Hepatobiliary Pancreat Dis Int (2018).

Cherblanc FL, Chapman KL, Brown R, Fuchter MJ. “Chaetocin is a nonspecific inhibitor of histone lysine methyltransferases”. Nat Chem Biol. 9:136–137 (2013).

Cherng CH, Lee KC, Chien CC, Chou KY, Cheng YC, Hsin ST, Lee SO, Shen CH, Tsai RY, Wong CS.” Baicalin ameliorates neuropathic pain by suppressing HDAC1 expression in the spinal cord of spinal nerve ligation rats”. J Formos Med Assoc 113(8):513-20 (2014).

Chiechio S, Zammataro M, Morales ME, Busceti CL, Drago F, Gereau RW 4th, Copani A, Nicoletti F.” Epigenetic modulation of mGlu2 receptors by histone deacetylase inhibitors in the treatment of inflammatory pain”. Mol Pharmacol 75(5):1014-20 (2009).

Chiu IM, von Hehn CA and Woolf CJ. “Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology”. Nature Neuroscience 15(8):1063-1067 (2012).

Chou C, Shrestha S, Yang C, Chang N, Lin Y, Liao K , Huang W, Sun T, Tu S, Lee W, Chiew M,Tai, Wei T, Tsai T, Huang H, Wang C, Wu H, Ho S, Chen P, Chuang C, Hsieh P, Wu Y, Chen W, Li M, Wu Y, Huang X, Ng F, Buddhakosai W, Huang P, Lan P, Huang C, Weng S, Cheng Y, Liang C, Hsu W, Huang H.”miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions”. Nucleic Acids Res 4; 46 (2018).

Chuang YH, Paul KC, Bronstein JM, Bordelon Y, Horvath S, Ritz B.” Parkinson's disease is associated with DNA methylation levels in human blood and saliva”. Genome Med 30; 9(1):76 (2017).

Chugh PE, Sin SH, Ozgur S, Henry DH, Menezes P, Griffith J, Eron JJ, Damania B, Dittmer DP.” Systemically circulating viral and tumor-derived microRNAs in KSHV-associated malignancies”. PLoS Pathog 9(7) (2013).

Cioce M and Lamond AI.” Cajal bodies: a long history of discovery”. Annu RevCell Dev Biol 21:105-31 (2005).

Cioce M, Boulon S, Matera AG, Lamond AI.” UV-induced fragmentation of Cajal bodies”. J Cell Biol 6:175(3):401-13 (2006).

Colleoni M, Sacerdote P.”Murine models of human neuropathic pain”. 10, 1802: 924-933 (2010).

Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, Freeman R, Truini A, Attal N, Finnerup NB, Eccleston C, Kalso E, Bennett DL, Dworkin RH and Raja SN.”Neuropathic pain”. Nat Rev Dis Primers 16; 3 (2017).

Coppedè F, Stoccoro A, Mosca L, Gallo R, Tarlarini C, Lunetta C, Marocchi A, Migliore L, Penco S.” Increase in DNA methylation in patients with amyotrophic lateral sclerosis carriers of not fully penetrant SOD1 mutations”. Amyotroph Lateral Scler Frontotemporal Degener 19(1-2):93-101 (2018).

Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B. “Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease”. Circulation Cardiovascular Genetics 3(6):499-506 (2010).

Costigan M, Scholz J and Woolf CJ. “Neuropathic pain: a maladaptive response of the nervous system to damage”. Annual Review of Neuroscience 32:1-32 (2009).

Cremer T, Cremer M, Dietzel S, Müller S, Solovei I, Fakan S.” Chromosome territories: a functional nuclear landscape”. Curr Opin Cel Biol 18(3):307-16 (2006).

Cremer T, Cremer C.” Rise, fall and resurrection of chromosome territories: a historical perspective. Part II. Fall and resurrection of chromosome territories during the 1950s to 1980s. Part III. Chromosome territories and the functional nuclear architecture: experiments and models from the 1990s to the present”. Eur J Histochem 50(4):223-72 (2006).

Cui W, Hu SX, Tang ZY, Hu KQ.” In-vivo effects and mechanisms of celecoxib-reduced growth of cyclooxygenase-2 (COX-2)-expressing versus COX-2-deleted human HCC xenografts in nude mice”. Anticancer Drugs 19(9):891-7 (2008).

Cullen BR. ¨Transcription and processing of human microRNA¨. Molecular Cell 16(6):861-865 (2004).

Dado RJ, Law PY, Loh HH, Elde R.” Immunofluorescent identification of a delta-opioid receptor on primary afferent nerve terminals”. Neuroreport 5:341-344 (1993).

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA. “Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia”. Nature 405(6783):183-187 (2000).

Dayeh T, Volkov P, Salö S, Hall E, Nilsson E, Olsson AH, Kirkpatrick CL, Wollheim CB, Eliasson L, Rönn T, Bacos K, Ling C.” Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion”. PLoS Genet 6; 10(3) (2014).

Decosterd,I, and Woolf ,C.K. “Spared nerve injury: an animal model of persistent peripheral neuropathic pain”. Pain 87, 149-158.doi:10.1016/S0304-3959 (00) (2000).

Denk F and McMahon S. “Chronic Pain: Emerging Evidence for the Involvement of Epigenetics”. Neuron, 73(3):435-444 (2012).

Denk F, Huang W, Sidders B, Bithell A, Crow M, Grist J, Sharma S, Ziemek D, Rice AS, Buckley NJ, McMahon SB.” HDAC inhibitors attenuate the

development of hypersensitivity in models of neuropathic pain". 154(9):1668-79 (2013).

Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. "Processing of primary microRNAs by the Microprocessor complex". Nature 432(7014):231-235 (2004).

Descalzi G, Ikegami D, Ushijima T, Nestler EJ, Zachariou V and Narita M." Epigenetic mechanisms of chronic pain". Trends Neuroscience 38(4):237-46 (2015).

Dharmshaktu P, Tayal V, Kalra BS." Efficacy of antidepressants as analgesics: a review". J Clin Pharmacol 52(1):6-17 (2012).

Dluzen DF, Sun D, Salzberg AC, Jones N, Bushey RT, Robertson GP and Lazarus P" Regulation of UDP-glucuronosyltransferase 1A1 expression and activity by microRNA 491-3p". J Pharmacol Exp Ther 348(3):465-77 (2014).

D'Mello R and Dickenson AH. "Spinal cord mechanisms of pain". Br J Anaesth 101(1):8-16 (2008).

Doehring A, Oertel B.G, Sittl R, Lotsch J. "Chronic opioid use is associated with increased DNA methylation correlating with increased clinical pain". Pain 154, 15e23 (2013).

Dong L, Ren H." Blood-based DNA Methylation Biomarkers for Early Detection of Colorectal Cancer". J Proteomics Bioinform 11(6):120-126 (2018).

Doron-Mandel E, Fainzilber M, Terenzio M. "Growth control mechanisms in neuronal regeneration". FEBS letters 589, 14:1669-1677 (2015).

Dostrovsky JO and Craig AD in Wall and Melzack´s textbook of pain. "Ascending proyection systems". McMahon SB, Kolzenburg M (Elsevier, Philadelphia), 187-203 (2006).

Dragon F, Gallagher JE, Compagnone-Post PA, Mitchell BM, Porwancher KA, Wehner KA, Wormsley S, Settlage RE, Shabanowitz J, Osheim Y, Beyer AL, Hunt DF, Baserga SJ." A large nucleolar U3

ribonucleoprotein required for 18S ribosomal RNA biogenesis". Nature 27; 417(6892):697-70 (2002).

Du J, Johnson L, Jacobsen S, Patel D." DNA methylation pathways and their crosstalk with histone methylation". Nat Rev Mol Cell Biol 16 (9):519-532 (2015).

Dubin AE and Patapoutian A. "Nociceptors: the sensors of the pain pathway". The Journal Clinical Investigation 120(11):3760-3772 (2010).

Echeverry S, Shi XQ, Haw A, Liu H, Zhang ZW, Zhang J." Transforming growth factor-beta1 impairs neuropathic pain through pleiotropic effects". Mol Pain 27; 5:16 (2009).

Echeverry S, Wu Y and Zhang J. "Selectively reducing cytokine/chemokine expressing macrophages in injured nerves impairs the development of neuropathic pain". Experimental Neurology 240:205-218 (2013).

Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C." Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells". Nucleic Acids Res 24; 10(8):2709-21 (1982).

Elramah S, Landry M, and Favereaux A." MicroRNAs regulate neuronal plasticity and are involved in pain mechanisms". Front Cell Neurosci 8:31 (2014).

Esteller M, Corn PG, Baylin SB, Herman JG." A gene hypermethylation profile of human cancer". Cancer Res 61: 3225-9 (2001).

Eymery A, Callanan M and Vourc'h C. The secret message of heterochromatin: new insights into the mechanisms and function of centromeric and pericentric repeat sequence transcription. Int J Dev Biol. 53:259-68 (2009).

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA. "Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation". Mol Cell 35: 868–880 (2009).

Farley M and Watkins TA.” Intrinsic neuronal stress response pathways in injury and disease”. Annu Rev Pathol 13:93-116 (2018).

Favereaux A, Thoumine O, Bouali-Benazzoua R, Roques V, Papon MA, Salam SA, Drutel G, Leger C, Calas A, Nagy F and Landry M. “Bidiretional integrative regulation of Cav 1.2 calcium channel by microRNA mir 103: role in pain”. The EMBO Journal 30:3830-3841 (2011).

Fehlmann T, Ludwig N, Backes C, Meese E, Keller A.” Distribution of microRNA biomarker candidates in solid tissues and body fluids”. RNA Biol 13(11):1084-1088 (2016).

Felsenfeld G and Groudine M.” Controlling the double helix”. Nature. 23; 421:448-53 (2003).

Feng L, Jing L, Han J, Wang G, Liu Y, Zhang X, Wang Y, Wang F, Ma H, Liu Y.” MicroRNA 486-3p directly targets BIK and regulates apoptosis and invasion in colorectal cancer cells”. Onco targers ther 6; 11:8791-8801 (2018).

Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Röxe T, Müller-Ardogan M, Bonauer A, Zeiher AM, Dimmeler S. “Circulating microRNAs in patients with coronary artery disease”. Circulation Research 107(5):677-684 (2010).

Fields HK, Basbaum A and Heinrich RL.“Central nervous system mechanisms of pain modulation”. En McMahon SB, Koltzenburg M, editors. Wall and Melzack´s textbook of pain, 5a edition. Philadelphia: Elsevier: 125-142 (2006).

Fierz B, Kilic S, Hieb AR, Luger K, Muir TW.” Stability of nucleosomes containing homogenously ubiquitylated H2A and H2B prepared using semisynthesis”. J Am Chem Soc 5; 134(48):19548-21 (2012).

Filipowicz W, Sonenberg N.” The long unfinished march towards understanding microRNA-mediated repression”. RNA 21(4):519-524 (2015).

Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpää M, Hansson P, Jensen TS, Kamerman PR, Lund

K, Moore A12, Raja SN, Rice AS, Rowbotham M, Sena E, Siddall P, Smith BH and Wallace M. "Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis". Lancet Neurol 14(2):162-73 (2015).

Flórez, J. "El tratamiento farmacológico del dolor". Ars Medica. (2007).

Foye C, Yan IK, David W, Shukla N, Habboush Y, Chase L, Ryland K, Kesari V, Patel T." Comparison of miRNA quantitation by Nanostring in serum and plasma samples". PLoS One 6; 12(2) (2017).

França GS, Vibranovski MD and Galante PA." Host gene constraints and genomic context impact the expression and evolution of human microRNAs". Nature Communications 25; 7 (2016).

Frost R and Olson EN. "Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs". Proceedings of the National Academy Sciences 108(52):21075-21080(2011).

Fu J, Xu X, Kang L. "miR-30a suppresses breast cancer cell proliferation and migration by targeting Eya2". Biochem Biophys Res Commun 445: 314–319 (2014).

Fuks F." DNA methylation and histone modifications: teaming up to silence genes". Curr Opin Genet Dev 15(5):490-5 (2005).

Gall JG." **Cajal** bodies: the first 100 years". Annu Rev Cell Dev Biol 16; 273-300 (2000).

Gambacciani C, Kusmic C, Chiavacci E, Meghini F, Rizzo M, Mariani L and Pitto L. "miR-29a and miR-30c negatively regulate DNMT3a in cardiac ischemic tissues: Implications for cardiac remodeling". microRNA diagnostics and therapeutics 35-45 (2013).

Gangaraju VK and Lin H. "MicroRNAs: key regulators of stem cells". Nature Reviews Molecular Cell Biology 10(2):116–125 (2009).

García R, Villar AV, Cobo M, Llano M, Martín-Durán R, Hurlé MA, Nistal JF." Circulating levels of miR-133a predict the regression potential of left ventricular hypertrophy after valve replacement surgery in patients with aortic stenosis". J Am Heart Assoc 15; 2(4) (2013).

García R, Nistal JF, Merino D, Price NL, Fernández-Hernando C, Beaumont J, González A, Hurlé MA, Villar AV.”P-SMAD2/3 and DICER promote pre-miR-21 processing during pressure overload-associated myocardial remodeling”. 1852(7):1520-30 (2015).

García-Esparcia P, Hernández-Ortega K, Koneti A, Gil L, Delgado-Morales R, Castaño E, Carmona M, Ferrer I.” Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease”. Acta Neuropathol Commun 1; 3; 76 (2015).

García-Porrero JA, Hurlé JM. “Neuroanatomía Humana”. Editorial médica Panamericana (2015).

Garriga J, Laumet G, Chen SR, Zhang Y, Madzo J, Issa JJ, Pan HL and Jelinek J. “Nerve Injury-Induced Chronic Pain Is Associated with Persistent DNA Methylation Reprogramming in Dorsal Root Ganglion”. J Neuroscience 4:38 (27) (2018).

Gatto S, Gagliardi M, Franzese M, Leppert S, Papa M, Cammisa M, Grillo G, Velasco G, Francastel C, Toubiana S, D'Esposito M, Angelini C, Matarazzo MR.” ICF-specific DNMT3B dysfunction interferes with intragenic regulation of mRNA transcription and alternative splicing”. Nucleic Acids Research 2; 45(10):5739-5756 (2017).

Genda Y, Arai M, Ishikawa M, Tanaka S, Okabe T and Sakamoto A. “microRNA changes in the dorsal horn of the spinal cord of rats with chronic constriction injury: a TaqMan(R) Low Density Array study”. Int. J. Mol. Med. 31 129–137 (2013).

Geuna S, Perroteau I, Tos P, Battiston B.” Peripheral nerve repair is no longer a matter of surgical reconstruction only”. Int Rev Neurobiol 109 (2013).

Gheinani AH, Burkhard FC, Monastyrskaya K. “Deciphering microRNA code in pain and inflammation: lessons from bladder pain syndrome”. Cell Molecular Life Science 70(20):3773-3789 (2013).

Gilron I, Baron R and Jensen T.” Neuropathic pain: principles of diagnosis and treatment”. Mayo Clin Proc 90(4):532-45 (2015).

Goldberg DS and McGee SJ. "Pain as a global public health priority". BMC Public Health 6; 11:770 (2011).

Goldie BJ and Cairns MJ. "Post-transcriptional trafficking and regulation of neuronal gene expression". Molecular Neurobiology 45: 99–108 (2012).

Gong Q, Lu Z, Huang Q, Ruan L, Chen J, Liang Y, Wang H, Yue Y, Feng S." Altered microRNAs expression profiling in mice with diabetic neuropathic pain". Biochem Biophys Res Commun 9; 456(2):615-20 (2015).

Grace PM, Rolan PE and Hutchinson MR. "Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain". Brain Behavior and Immunity 25(7):1322-1332 (2011).

Grace PM, Hutchinson MR, Maier SF, Watkins LR." Pathological pain and the neuroimmune interface". Nat Rev Immunol 14(4):217-31 (2014).

Gräff J, Kim D, Dobbin MM and Tsai LH." Epigenetic regulation of gene expression in physiological and pathological brain processes". Physiol Rev 91(2):603-49 (2011).

Greer EL and Shi Y. "Histone methylation: a dynamic mark in health, disease and inheritance". Nat Rev Genet 3; 13(5):343-57 (2012).

Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. "MicroRNA targeting specificity in mammals: determinants beyond seed pairing". Molecular Cell 27(1):91-105 (2007).

Gryshkova V, Fleming A, McGhan P, De Ron P, Fleurance R, Valentin JP and Nogueira da Costa A." miR-21-5p as a potential biomarker of inflammatory infiltration in the heart upon acute drug-induced cardiac injury in rats". Toxicol Lett; 31-38 (2018).

Gui Y, Liu H, Zhang L, Lv W, Hu X." Altered microRNA profiles in cerebrospinal fluid exosome in Parkinson disease and Alzheimer disease". Oncotarget 10; 6(35) (2015).

Guo K, Liang Z, Li F, Wang H." Comparison of miRNA and gene expression profiles between metastatic and primary prostate cancer". Oncol Lett 14(5):6085-6090 (2017).

Guz J, Foksiński M and Oliński R.” [Mechanism of DNA methylation and demethylation--its role in control of genes expression]”. Postepy Biochem 56(1):7-15 (2010).

Gwak Y, Hulsebosch C and Leem J. “Neuronal-Glial interactions maintain chronic neuropathic pain after spinal cord injury”. Neural Plast (2017).

Hahn CK, Ross KN, Warrington IM, Mazitschek R, Kanegai CM, Wright RD, Kung AL, Golub TR, Stegmaier K.” Expression-based screening identifies the combination of histone deacetylase inhibitors and retinoids for neuroblastoma differentiation”. Proc Natl Acad Sci USA 15; 105(28):9751-6 (2008).

Hammond SM. “An overview of microRNAs”. Adv Drug Deliv Rev 29; 87:3-14 (2015).

Han X, Zhen S, Ye Z, Lu J, Wang L, Li P, Li J, Zheng X, Li H, Chen W, Li X and Zhao L.” A Feedback Loop Between miR-30a/c-5 and DNMT1 mediates cisplatin resistance in ovarian cancer cells”. Cell Physiol Biochem 41(3):973-986 (2017).

Hanakawa T.” Neural mechanisms underlying deafferentation pain: a hypothesis from a neuroimaging perspective”. J Orthop Sci 17 (3):331-5 (2012).

Hand NJ, Master ZR, EauClaire SF, Weinblatt DE, Matthews RP, Friedman JR. “The microRNA-30 family is required for vertebrate hepatobiliary development”. Gastroenterology. 136(3): 1081–1090 (2009).

Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, Jocham D, Warnecke JM, Sczakiel G. “A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer”. Urologic Oncology 28(6):655-661 (2010).

Harb H, Amarasekera M, Ashley S, Tulic MK, Pfefferle PI, Potaczek DP, Martino D, Kesper DA, Prescott SL, Renz H.” Epigenetic Regulation in Early Childhood: A Miniaturized and Validated Method to Assess Histone Acetylation”. Int Arch Allergy Immunol 168(3):173-81 (2015).

Hayati A and Badariah C. "The brain in Pain". Malays J Med Sci, 21:46-54 (2014).

He L y, Hannon GJ. "MicroRNAs: small RNAs with a big role in gene regulation". Nature Review Genetics 5(7):522–531 (2004).

He X, Chen T, Zhu J." Regulation and function of DNA methylation in plants and animals". Cell Res 21(3):442-465 (2011).

Hébert SS, De Strooper B. "Alterations of the MicroRNAs network cause neurodegenerative disease". Trends in Neurosciences 32(4):199-206 (2009).

Heard E, Clerc P, Avner P. "X-chromosome inactivation in mammals". Annu Rev Genet 31,571-610 (1997).

Hernández-Ortega K, Garcia-Esparcia P, Gil L, Lucas JJ, Ferrer I." Altered Machinery of Protein Synthesis in Alzheimer's: From the Nucleolus to the Ribosome". Brain Pathol 26(5):593-605 (2016).

Hernandez-Verdun D, Roussel P, Thiry M, Sirri V and Lafontaine DL." The nucleolus: structure/function relationship in RNA metabolism". Wiley nterdiscio Rev RNA 1(3):415-431 (2010).

Hobert O. "Gene regulation by transcription factors and microRNAs". Science, 319(5871):1785-6 (2008).

Hocher A, Ruault M, Kaferle P, Descrimes M, Garnier M, Morillon A and Taddei A." Expanding heterochromatin reveals discrete subtelomeric domains delimited by chromatin landscape transitions". Genome Res 28(12):1867-1881 (2018).

Hori Y, Goto G, Arai-Iwasaki M, Ishikawa M, Sakamoto A. "Differential expression of rat hippocampal microRNAs in two rat models of chronic pain. International Journal of Molecular Medicine 32(6):1287-1292 (2013).

Hsu E and Cohen SP." Postamputation pain: epidemiology, mechanisms, and treatment". J Pain Res 6; 121-36 (2013).

Hu Y, Tang Z, Jiang B, Chen J, Fu Z.” miR-198 functions as a tumor suppressor in breast cancer by targeting CUB domain-containing protein 1”. Oncol Lett 13(3):1753-1760 (2017).

Huang Y, Li X, Tao G, Zhu T, Lin J.” Comparing serum microRNA levels of acute herpes zoster patients with those of postherpetic neuralgia patients”. Medicina (Baltimore), 96(8) (2017).

Huang W, Su G, Huang X, Zou A, Wu J, Yang Y, Zhu Y, Liang S, Li D, Ma F, Guo L.” Long noncoding RNA PCAT6 inhibits colon cancer cell apoptosis by regulating anti-apoptotic protein ARC expression via EZH2”. Cell cycle 19 (2018).

Huo X, Mao Ying Q, Zhou W, Danzter R, Heijnen CJ, Kavelaars A. “Intrathecal administration of microRNA-124 alleviates both neuropathic pain and depression like behavior in mice”. Elsevier, Brain Behavior and Immunity 32:19 (2013).

Hyun K, Jeon J, Park K, Kim J.” Writing, erasing and reading histone lysine methylations”. Exp Mol Med 28; 49(4) (2017).

Im YB, Jee MK, Choi JI, Cho HT, Kwon OH, Kang SK. “Molecular targeting of NOX4 for neuropathic pain after traumatic injury of the spinal cord”. Cell Death Dis, 3 (2012).

Imai S, Saeki M, Yanase M, Horiuchi H, Abe M, Narita M, Kuzumaki N, Suzuki T and Narita M. “Change in microRNAs associated with neuronal adaptive responses in the nucleus accumbens under neuropathic pain”. *J*. Neurosci*.* 31(2011).

Ikoma A, Rukwied R, Stander S, Steinhoff M, Miyachi Y, Schmelz M. “Neuronal sensitization for histamine-induced itch in lesional skin of patients with atopic dermatitis”. Arch Dermatol 139:1455–1458 (2003).

Ito S, D’Alessio A, Olena V. Taranova, Kwonho Hong, Lawrence C. Sowers, Yi Zhang.” Role of Tet proteins in 5mC to 5hmC conversion, ES cell self-renewal, and ICM specification”. Nature 466(7310):1129-1133 (2010).

Ito Y, Shibata N, Saito K, Kobayashi M, Osawa M.” New insights into the pathogenesis of spinal muscular atrophy”. Brain Dev 33(4):321-31 (2011).

Jackson J, Lindroth A, Cao X and Jacobsen,S.E. “Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase”. Nature, 416, 556–560 (2002).

Jeltsch A. “Molecular enzymology of mammalian DNA methyltransferases”. Curr.Top.Microbiol.Immunol 301, 203-225 (2006).

Jiang BC, He LN, Wu XB, Shi H, Zhang WW, Zhang ZJ, Cao DL, Li CH, Gu J, Gao YJ.” Promoted Interaction of C/EBPα with Demethylated Cxcr3 Gene Promoter Contributes to Neuropathic Pain in Mice”. J Neurosci 18; 37(3):685-700 (2017).

Jiang BC, Zhang WW, Yang T, Guo CY, Cao DL, Zhang ZJ and Gao YJ.” Demethylation of G-Protein-Coupled Receptor 151 Promoter Facilitates the Binding of Krüppel-Like Factor 5 and Enhances Neuropathic Pain after Nerve Injury in Mice”. J Neurosci 5; 38(49):10535-10551 (2018).

Jiao S, Liu Y, Yao Y, Teng J.” miR-124 promotes proliferation and differentiation of neuronal stem cells through inactivating Notch pathway”. Cell Biosci 11; 7:68 (2017).

Jin XF, Wu N, Wang L, Li J. “Circulating microRNAs: a novel class of potential biomarkers for diagnosing and prognosing central nervous system diseases”. Cellular and Molecular Neurobiology 33(5):601-613 (2013).

Jin J, Kim SN, Liu X, Zhang H, Zhang C, Seo JS, Kim Y, Sun T.” miR-17-92 Cluster Regulates Adult Hippocampal Neurogenesis, Anxiety, and Depression”. Cell Rep 9; 16(6):1653-1663 (2016).

Jin J, Lian T, Gu C, Yu K, Gao Y, and Su X. “The effects of cytosine methylation on general transcription factors”. Scientific reports 6 (2016).

Johnson IP and Sears TA. "Target-dependence of sensory neurons: an ultrastructural comparison of axotomised dorsal root ganglion neurons with allowed or denied reinnervation of peripheral targets". Neuroscience 228:163-78 (2013).

Johnson AC and Greenwood-Van Meerveld B. "Stress-induced pain: a target for the development of novel therapeutics". J Pharmacol Exp Ther 351:327-335 (2014).

Jordan BA, Fernholz BD, Khatri L, Ziff EB." Activity-dependent AIDA-1 nuclear signaling regulates nucleolar numbers and proteinsynthesis in neuron". Nat Neurosci 10(4):427-35 (2007).

Julius D and Basbaum AI. "Molecular mechanisms of nociception". Nature 413(6852):203-210 (2001).

Kami K, Taguchi S, Tajima F, Senba E." Histone Acetylation in Microglia Contributes to Exercise-Induced Hypoalgesia in Neuropathic Pain Model Mice". J Pain 17(5):588-99 (2016).

Kan Q, Su Y, Yang H." MicroRNA-335 is downregulated in papillary thyroid cancer and suppresses cancer cell growth, migration and invasion by directly targeting ZEB2". 14 (2017).

Kandel E. "Principles of Neural Science". Edited, James H. Schwartz, Thomas M. Jessell, Steven A. Siegelbaum, A Hudspeth and Sarah Mack. 24: Pain. 5th Edition (2000).

Kang M, Liu X, Fu Y, Timothy Garvey W." Improved systemic metabolism and adipocyte biology in miR-150 knockout mice". Metabolism 83; 139-148 (2018).

Kaniskan HÜ, Martini ML, Jin J." Inhibitors of Protein Methyltransferases and Demethylases". Chem Rev 14; 118(3):989-1068 (2018).

Karpova NN." Role of BDNF epigenetics in activity-dependent neuronal plasticity". Neuropharmacology 76 (2014).

Keshet I, Lieman-Hurwitz J, Cedar H." DNA methylation affects the formation of active chromatin". Cell 28:44(4):535-43 (1986).

Khvorova A, Reynolds A, Jayasena SD. "Functional siRNAs and miRNAs exhibit strand bias". Cell Journal Home 115(2):209–216 (2003).

Kiguchi N, Kobayashi Y, Saika F, Kishioka S.” Epigenetic upregulation of CCL2 and CCL3 via histone modifications in infiltrating macrophages after peripheral nerve injury”. Cytokine 64(3):666-72 (2013).

Kiguchi N, Kobayashi Y, Kadowaki Y, Fukazawa Y, Saika F, Kishioka S.” Vascular endothelial growth factor signaling in injured nerves underlies peripheral sensitization in neuropathic pain”. J Neurochem 129(1):169-78 (2014).

Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, Hannon G and Abeliovich A.” A MicroRNA feedback circuit in midbrain dopamine neurons”. Science 31; 317(5842):1220-4 (2007).

Kim Y, Zhang Y, Pang K, Kang H, Park H, Lee Y, Lee B, Lee HJ, Kim WK, Geum D, Han K.” Bipolar Disorder Associated microRNA, miR-1908-5p, Regulates the Expression of Genes Functioning in Neuronal Glutamatergic Synapses”. Exp Neurobiol 25(6):296-306 (2016).

Kloosterman WP, Plasterk RH. “The diverse functions of microRNAs in animal development and disease”. Developmental Cell 11(4):441–450 (2006).

Kohli RM and Zhang Y.” TET enzymes, TDG and the dynamics of DNA demethylation”. Nature 24; 502(7472):472-9 (2013).

Kohno T, Ji RR, Ito N, Allchorne AJ, Befort K, Karchewski LA and Woolf CJ. “Peripheral axonal injury results in reduced mu opioid receptor pre- and post-synaptic action in the spinal cord”. Pain 117(1-2):77-87 (2005).

Kouzarides T.” SnapShot: Histone-modifying enzymes”. Cell 16; 131 (4):822 (2007).

Kozomara A and Griffiths-Jones S. “miRBase: integrating microRNA annotation and deep-sequencing data”. Nucleic Acids Research 39:D152-157(2011).

Kress M, Hüttenhofer A, Landry M, Kuner R, Favereaux A, Greenberg D, Bednarik J, Heppenstall P, Kronenberg F, Malcangio M, Rittner H, Uçeyler N, Trajanoski Z, Mouritzen P, Birklein F, Sommer C, Soreq H.

"MicroRNAs in nociceptive circuits as predictors of future clinical applications". Front Molecular Neuroscience 6:33 (2013).

Krol J, Loedige I, Filipowicz W. "The widespread regulation of microRNA biogenesis, function and decay". Nature Reviews Genetics 11(9):597-610 (2010).

Kukkar A, Singh N, Jaggi AS." Attenuation of neuropathic pain by sodium butyrate in an experimental model of chronic constriction injury in rats". J Formos Med Assoc 113(12):921-8 (2014).

Kusuda R, Cadetti F, Ravanelli MI, Sousa TA, Zanon S, De Lucca FL, and Lucas G. "Differential expression of microRNAs in mouse pain models". Mol. Pain 7:17 (2011).

Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, Watanabe S, Baba O, Kojima Y, Shizuta S, Imai M, Tamura T, Kita T, Kimura T. "Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage". Circulation Cardiovascular Genetics 4(4):446-454 (2011).

Kuypers NJ, Bankston AN, Howard RM, Beare JE, Whittemore SR." Remyelinating Oligodendrocyte Precursor Cell miRNAs from the Sfmbt2 Cluster Promote Cell Cycle Arrest and Differentiation". J Neurosci 3; 36(5):1698-710 (2016).

Kynast KL, Russe OQ, Geisslinger G, Niederberger E. "Novel findings in pain processing pathways: implications for miRNAs as future therapeutic targets". Expert Review of Neurotherapeutics 13(5):515-525 (2013).

Lafarga M, Andres MA, Berciano MT, Maquiera E." Organization of nucleoli and nuclear bodies in osmotically stimulated supraoptic neurons of the rat". J Comp Neurol 15; 308(3):329-39 (1991).

Lafarga M, Casafont I, Bengoechea R, Tapia O, Berciano MT." Cajal's contribution to the knowledge of the neuronal cell nucleus". Chromosoma 118(4):437-43 (2009).

Lafarga, Tapia O, Romero AM and Berciano MT. "Cajal bodies in neurons". RNA Biol, 14(6):712-725 (2017).

Lafita-Navarro MC, Blanco R, Mata-Garrido J, Liaño-Pons J, Tapia O, García-Gutiérrez L, García-Alegría E, Berciano MT, Lafarga M, León J.” MXD1 localizes in the nucleolus, binds UBF and impairs rRNA synthesis”. Oncotarget 25; 7(43):69536-69548 (2016).

Lafontaine DL.” A 'garbage can' for ribosomes: how eukaryotes degrade their ribosomes”.Trends Biochem Sci 35(5):267-77 (2010).

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. “Identification of novel genes coding for small expresed RNAs”. Science 294(5543):853-858 (2001).

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. “Identification of tissue specific microRNAs from mouse”. Current Biology 12(9):735-739 (2002).

Lantero A, Tramullas M, Díaz A and Hurlé MA.” Transforming growth factor-β in normal nociceptive processing and pathological pain models”. Mol Neurobiol 45(1):76-86 (2012).

Larsen LK, Jakobsen JS, Abdul-Al A, Guldberg P.” Noninvasive Detection of High Grade Prostate Cancer by DNA Methylation Analysis of Urine Cells Captured by Microfiltration”. J Urol 200(4):749-757 (2018).

Latremoliere A and Woolf CJ. “Central sensitization: a generator of pain hypersensitivity by central neural plasticity”. Journal Pain 10(9):895-926 (2009).

Lau NC, Lim LP, Weinstein EG, Bartel DP. “An abundant class of tiny RNAs with probable regulatory roles in C. Elegans”. Science 294(5543):858-862 (2001).

Laumet G, Garriga J, Chen SR, Zhang Y, Li DP, Smith TM, Dong Y, Jelinek J, Cesaroni M, Issa JP, Pan HL.” G9a is essential for epigenetic silencing of K (+) channel genes in acute-to-chronic pain transition”. Nat Neurosci 18(12):1746-55 (2015).

Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL.” Dynamic changes in the human methylome during differentiation”. Genome Res 20(3):320-31 (2010).

Lee RC, Feinbaum RL, Ambros V. "The C.Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14". Cell 75(5):843-854 (1993).

Lee RC and Ambros V. "An extensive class of small RNAs in Caenorhabditis Elegans". Science 294(5543):862-864 (2001).

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. "The nuclear RNase Drosha initiates microRNA processing". Nature 425(6956):415–419 (2003).

Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim N. "MicroRNA genes are transcribed by RNA polymerase II". EMBO Journal 23(20):4051–4060 (2004).

Lee K, Na W, Lee JY, Na J, Cho H, Wu H, Yune TY, Kim WS, Ju BG." Molecular mechanism of Jmjd3-mediated interleukin-6 gene regulation in endothelial cells underlying spinal cord injury". J Neurochem 122(2):272-82 (2012).

Lee J, Lee MS, Jeoung DI, Kim YM, Lee H." Promoter CpG-Site Methylation of the KAI1 Metastasis Suppressor Gene Contributes to Its Epigenetic Repression in Prostate Cancer". Prostate 77(4):350-360 (2017).

Leggio L, Vivarelli S, L'Episcopo F, Tirolo C, Caniglia S, Testa N, Marchetti B, Iraci N." microRNAs in Parkinson's Disease: From Pathogenesis to Novel Diagnostic and Therapeutic Approaches". Int J Mol Sci 18(12); 2698 (2017).

Lehnertz B, Ueda Y, Derijck AA, Braunschweig U, Perez-Burgos L, Kubicek S, Chen T, Li E, Jenuwein T, Peters AH." Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin". Curr Biol 15; 13(14):1192-200 (2003).

Leoni F, Fossati G, Lewis EC, Lee JK, Porro G, Pagani P, Modena D, Moras ML, Pozzi P, Reznikov LL, Siegmund B, Fantuzzi G, Dinarello CA, Mascagni P." The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines *in vitro* and systemic inflammation in vivo". Mol Med 11(1-12):1-15 (2005).

Lewis BP, Burge CB, Bartel DP. "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets". Cell 120(1):15-20 (2005).

Li X., Jin P. "Roles of small regulatory RNAs in determining neuronal identity. Nat. Rev. Neurosci". 11, 329–338 (2010).

Li X, Gibson G, Kim JS, Kroin J, Xu S, Van Wijnen AJ, Im HJ. "MicroRNA-146a is linked to pain related pathophysiology of osteoarthritis". Gene 480(1-2):34-41 (2011).

Li ZF, Liang YM, Lau PN, Shen W, Wang DK, Cheung WT, Xue CJ, Poon LM, Lam YW." Dynamic localisation of mature microRNAs in Human nucleoli is influen ced by exogenousgenetic materials". PLos One 6; 8(8) (2013).

Li E and Zhang Y." DNA methylation in mammals". Cold Spring Harb Perspect Biol 6(5) (2014).

Li L, Jiang H, L, Y, Guo Y. "Hydrogen sulfide protects spinal cord and induces autophagy via miR-30c in a rat model of spinal cord ischemia-reperfusion injury". Journal of Biomedical Science. 22(1), 50 (2015).

Li X, Wan Y, Tang S, Guan Y, Wei F and Ma D. "Maladaptative Plasticity and Neuropathic Pain. Neural Plast (2016).

Liang L, Lutz B, Bekker A and Tao Y. "Epigenetic regulation of chronic pain". Epigenomics 7, 235-245 (2015).

Liang L, Gu X, Zhao JY, Wu S, Miao X, Xiao J, Mo K, Zhang J, Lutz BM, Bekker A, Tao YX." G9a participates in nerve injury-induced Kcna2 downregulation in primary sensory neurons". Sci Rep 22; 6; 37704 (2016).

Liao JY1, Ma LM, Guo YH, Zhang YC, Zhou H, Shao P, Chen YQ, Qu LH." Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3' trailers". PLoS one 14; 5(5) (2010).

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM." Microarray analysis shows that some

microRNAs downregulate large numbers of target mRNAs". Nature 17; 433(7027):769-73 (2005).

Lin SL, Miller JD, Ying SY. "Intronic microRNA (miRNA)". Journal of Biomedicine and Biotechnology 2006(4):26818 (2006).

Lingel A, Simon B, Izaurralde E, Sattler M. "Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain". Nature Structural y Molecular Biology 11(6):576-577(2004).

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR." Human DNA methylomes at base resolution show widespread epigenomic differences". Nature.19; 462(7271):315-22 (2009).

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. "Argonaute 2 is the catalytic engine of mammalian RNAi". Science 305(5689):1437-1441 (2004).

Liu NK and Xu XM." MicroRNA in central nervous system trauma and degenerative disorders" Physiol Genomics 43(10): 571-580 (2011).

Lötsch J, Doehring A, Mogil J.S , Arndt T, Geisslinger G, Ultsch A. "Functional genomics of pain in analgesic drug development and therapy". Pharmacology y Therapeutics 139(1):60-70 (2013).

Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. "Nuclear export of microRNA precursors". Science 303(5654):95–98 (2004).

Luo M, Li R, Ren M, Chen N, Deng X, Tan X, Li Y, Zeng M, Yang Y, Wan Q, Wu J. "Hyperglycaemia-induced reciprocal changes in miR-30c and PAI-1 expression in platelets". Sci Rep. 2016 Nov 7; 6:36687 (2016).

Ma JB, Yuan YR, Meister G, Pei Y, Tuschl T, Patel DJ. "Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein". Nature 434(7033):666-670 (2005).

Machyna M, Heyn P and Neugebauer KM. "Cajal bodies: where form meets function".Wiley Interdiscio Rev RNA 4(1):17-34 (2013).

Madakashira B, Corbett L, Chi Zhang, Paoli P, Casement J, Mann J , Sadler K, Mann D. "Variant Histone H2afv reprograms DNA methylation during early zebrafish development". Epigenetics 12(9):811-824 (2017).

Mahrez W, Arellano MS, Moreno-Romero J, Nakamura M, Shu H, Nanni P, Köhler C, Gruissem W and Hennig L." H3K36ac Is an Evolutionary Conserved Plant Histone Modification That Marks Active Genes". Plant Physiology 170(3):1566-77 (2016).

Majer A, Caligiuri KA, Gale KK, Niu Y, Phillipson CS, Booth TF, Booth SA." Induction of Multiple miR-200/182 Members in the Brains of Mice Are Associated with Acute Herpes Simplex Virus 1 Encephalitis". PLoS One 3; 12(1) (2017).

Malcangio M." Microglia and chronic pain". Pain 157(5):1002-3 (2016).

Mallory A and Bouche N. "MicroRNA-directed regulation: to cleave or not to cleave". Trends in Plant Science 13(7):359-367 (2008).

Mao X, Hou T, Cao B, Wang W, Li Z, Chen S, Fei M, Hurren R, Gronda M, Wu D, Trudel S, Schimmer AD." The tricyclic antidepressant amitriptyline inhibits D-cyclin transactivation and induces myelomacell apoptosis by inhibiting histone deacetylases: in vitro and in silico evidence". Mol Pharmacol 79(4):672-80 (2011).

Masala A, Sanna S, Esposito S, Rassu M, Galioto M, Zinellu A, Carru C, Carrì MT, Iaccarino C, Crosio C." Epigenetic Changes Associated with the Expression of Amyotrophic Lateral Sclerosis (ALS) Causing Genes". Neuroscience 15; 390:1-11 (2018).

Massagué J." TGFβ signalling in context". Nat Rev Mol Cell Biol 13(10):616-30 (2012).

Massara L, Osuru HP, Oklopcic A, Milanovic D, Joksimovic SM, Caputo V, DiGruccio MR, Ori C, Wang G, Todorovic SM and Jevtovic-Todorovic V." General Anesthesia Causes Epigenetic Histone Modulation of c-Fos and Brain-derived Neurotrophic Factor, Target Genes Important for Neuronal Development in the Immature Rat Hippocampus". Anesthesiology 124 (6):1311-1327 (2016).

Massenet S, Bertrand E, Verheggen C.” Assembly and trafficking of box C/D and H/ACA snoRNPs”. RNA Biol 3; 14(6):680-692 (2017).

Mastronardi FG, Noor A, Wood DD, Paton T, Moscarello MA.” Peptidyl argininedeiminase 2 CpG island in multiple sclerosis white matter is hypomethylated”. J Neurosci Res 85(9); 2006-16 (2007).

Martin L. J. “Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism”. J. Neuropathol. Exp. Neurol. 58, 459–471 (1999).

Matkovich SJ, Wang W, Tu Y, Eschenbacher WH, Dorn LE, Condorelli G, Diwan A, Nerbonne JM, Dorn GW 2nd.” MicroRNA-133a protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts”. Circ Res 8; 106(1):166-75 (2010).

Matsumoto L, Takuma H, Tamaoka A, Kurisaki H, Date H, Tsuji S, Iwata A.” CpG demethylation enhances alpha-synuclein expression and affects the pathogenesis of Parkinson's disease”. Plos One 24; 5 (11) (2010).

Matsushita Y, Araki Y, Omotuyi O, Mukae T, Ueda H.” HDAC inhibitors restore C-fibre sensitivity in experimental neuropathic pain model”. Br J Pharmacol 170(5):991-998 (2013).

Mauck M, Van de Ven T, Shaw AD.” Epigenetics of chronic pain after thoracic surgery”. Curr Opin Anaesthesiol 27(1):1-5 (2014).

McDonald MK and Ajit SK. “MicroRNA biology and pain”. Progress in Molecular Biology and Translational Science 131: 215-249 (2015).

McKinnon PJ. “DNA repair deficiency and neurological disease”. Nat Rev Neurosci. 10:100–112 (2009).

Meister G, Landthaler M, Dorsett Y, Tuschl T.” Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing”. RNA 10(3):544-550 (2004).

Melzack R and Casey KL. “Sensory, motivational and central control determinants of chronic pain: A new conceptual model”. En: Kenshalo, DR. The Skin Senses. Springfield, Illinois, 432- 449 (1968).

Messemaker TC, Mikkers HMM, Huizinga TW, Toes REM, van der Helm-van Mil AHM, Kurreeman F.” Inflammatory genes TNFα and IL6 display no signs of increased H3K4me3 in circulating monocytes from untreated rheumatoid arthritis patients”. Genes Immun 18(3):191-196 (2017).

Messerschmidt D, Knowles B, Solter D. “DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos”. Genes Dev 28(8):812-828 (2014).

Meyer RA, Ringkamp M, Campbell JN, and Raja SN. “Peripheral mechanisms of nociception”. 5th edition. Elsevier, (2006).

Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E and Przewlocka B. “Importance of glial activation in neuropathic pain”. European Journal of Pharmacology 716(1-3):106-119 (2013).

Millán-Zambrano G, Santos-Rosa H, Puddu F, Robson SC, Jackson SP, Kouzarides T.” Phosphorylation of Histone H4T80 Triggers DNA Damage Checkpoint Recovery”. Mol Cell 15; 72(4):625-635 (2018).

Miller BH, Zeier Z, Xi L, Lanz TA, Deng S, Strathmann J, Willoughby D, Kenny PJ, Elsworth JD, Lawrence MS, Roth RH, Edbauer D, Kleiman RJ and Wahlestedt C.” MicroRNA-132 dysregulation in schizophrenia has implications for both neurodevelopment and adult brain function”. Proc Natl Acad Sci USA 21; 109(8):3125-30 (2012).

Milligan ED and Watkins LR.“Pathological and protective roles of glia in chronic pain”. Nature Reviews Neuroscience 10(1):23–36 (2009).

Mills S, Torrance N and Smith BH. “Identification and Management of Chronic Pain in Primary Care: a Review”. Curr Psychiatry Rep 18(2):22 (2016).

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, PogosovaAgadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. “Circulating microRNAs as stable blood-based markers for cancer detection”. Proceedings of the National Academy of Sciences 105(30):10513-10518 (2008).

Mo K, Wu S, Gu X, Xiong M, Cai W, Atianjoh FE, Jobe EE, Zhao X, Tu WF, Tao YX.” MBD1 Contributes to the Genesis of Acute Pain and Neuropathic

Pain by Epigenetic Silencing of Oprm1 and Kcna2 Genes in Primary Sensory Neurons". J Neurosci 14,38(46):9883-9899 (2018).

Momen-Heravi F, Saha B, Kodys K, Catalano D, Satishchandran A, Szabo G." Increased number of circulating exosomes and their microRNA cargos are potential novel biomarkers in alcoholic hepatitis". J Transl Med 12; 13:261 (2015).

Mongelard F and Bouvet P." Nucleolin: a multiFACeTed protein". Trends Cell Biol 17(2):80-6 (2006).

Moon LDF. "Chromatolysis: Do injured axons regenerate poorly when ribonucleases attack rough endoplasmic reticulum, ribosomes and RNA?" Dev Neurobiol. 78(10):1011-1024 (2018).

Moore MJ and Proudfoot NJ." Pre-mRNA processing reaches back to transcription and ahead to translation". Cell 20:136(4):688-700 (2009).

Moore RA, Wiffen PJ, Derry S, Toelle T, Rice AS." Gabapentin for chronic neuropathic pain and fibromyalgia in adults". Cochrane DAtabase Syst Rev 27(4) (2014).

Morera L, Lübbert M, Jung M." Targeting histone methyltransferases and demethylases in clinical trials for cancer therapy". Clin Epigenetics 24; 8:57 (2016).

Mulrane L, McGee SF, Gallagher WM, O'Connor DP." miRNA dysregulation in breast cancer". Cancer Res 15; 73(22):6554-62 (2013).

Naaman H, Rall G, Matullo C, Veksler-Lublinsky I, Shemer-Avni Y, Gopas J." MiRNA-124 is a link between measles virus persistent infection and cell division of human neuroblastoma cells". PLoS One 12(10) (2017).

Nagel S, Pommerenke C, Meyer C, Kaufmann M, Drexler HG and MacLeod RA. "Deregulation of polycomb repressor complex 1 modifier AUTS2 in T-cell leukemia". Oncotarget, 17 (29) (2016).

Nan X, Cross S, Bird A." Gene silencing by methyl-CpG-binding proteins". Novartis Found Symp 214:6-16 (1998).

Nelson PT, Wang WX, Wilfred BR, Wei A, Dimayuga J, Huang Q, Ighodaro E, Artiushin S, Fardo DW. "Novel human ABCC9/SUR2 brain-expressed transcripts and an eQTL relevant to hippocampal sclerosis of aging". J Neurochem. 134(6):1026-39(2015).

Niederberger E, Kynast K, Lötsch J, Geisslinger G." MicroRNAs as new players in the pain game". Pain 152(7):1455-8 (2011).

Niederberger E, Resch E, Parnham MJ and Geisslinger G." Drugging the pain epigenome". Nat Rev Neurol 13(7):434-447 (2017).

Nieto-Díaz M, Esteban FJ, Reigada D, Muñoz-Galdeano T, Yunta M, Caballero-López M, Navarro-Ruiz R, Del Águila A, Maza RM." MicroRNA dysregulation in spinal cord injury: causes, consequences and therapeutics". Front Cell Neurosci 25; 8:53 (2014).

Nizami ZF, Deryusheva S, Gall JG." Cajal bodies and histone locus bodies in Drosophila and Xenopus". Cold Spring Harb Symp Quant Biol 75:313-20 (2010).

Norcini M, Sideris A, Martin Hernandez LA, Zhang J, Blanck TJ, Recio-Pinto Ealdri." An approach to identify microRNAs involved in neuropathic pain following a peripheral nerve injur". Front Neurosci 29; 8:266 (2014).

Oakes C, La Salle S, Robaire B and Trasler J. "Evaluation of a Quantitative DNA methylation analysis techinique using methylation sensitive/dependent restriction enzymes and real-time PCR". Epigenetics.1:3, 146-152 (2016).

Okano M1, Bell DW, Haber DA, Li E." DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development". Cell 29; 99(3):247-57 (1999).

Olins AL and Olins DE." Spheroid chromatin units (v bodies)". Science 13:330-2 (1974).

O'Neill J, Brock C, Olesen AE, Andresen T, Nilsson M and Dickenson AH. "Unravelling the mystery of capsaicin: a tool to understand and treat pain". Pharmacological Reviews 64(4):939-971 (2012).

Orlova IA, Alexander GM, Qureshi RA, Sacan A, Graciano A, Barret JE, Schwartzman RJ, Ajit SK. "MicroRNA modulation in complex regional pain syndrome". Journal of Translational Medecine 9:195 (2011).

Ossipov MH, Dussor GO and Porreca F. "Central modulation of pain". Journal of Clinical Investigation 120(11):3779-3787 (2010).

Özata DM, Li X, Lee L, Liu J, Warsito D, Hajeri P, Hultman I, Fotouhi O, Marklund S, Ährlund-Richter L, Juhlin CC, Larsson C, Lui WO." Loss of miR-514a-3p regulation of PEG3 activates the NF-kappa B pathway in human testicular germ cell tumors". Cell Death Dis 4; 8(5) (2017).

Pacaud R, Sery Q, Oliver L, Vallette FM, Tost J and Cartron PF. "DNMT3L interacts with transcription factors to target DNMT3L/DNMT3B to specific DNA sequences: role of the DNMT3L/DNMT3B/p65-NFκB complex in the (de-methylation of TRAF1". Biochimie 104:36-49 (2014).

Paladini L, Fabris L, Bottai G, Raschioni C, Calin GA, Santarpia L." Targeting microRNAs as key modulators of tumor immune response". J Exp Clin Cancer Res 27; 35:103 (2016).

Palanca A, Casafont I, Berciano MT, Lafarga M." Reactive nucleolar and Cajal body responses to proteasome inhibition in sensory ganglion neurons". Biochim Biophys Acta 1842(6):848-59 (2014).

Pang RT, Leung CO, Ye TM, Liu W, Chiu PC, Lam KK, Lee KF, Yeung WS." MicroRNA-34a suppresses invasion through downregulation of Notch1 and Jagged1 in cervical carcinoma and choriocarcinoma cells". Carcinogenesis 31(6):1037-44 (2010).

Park NJ, Zhou H, Blastoff D, Henson BS, Kastratovic DA, Abemayor E, Wong DT. "Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection". Clinical Cancer Research 15(17):5473-5477 (2009).

Parker JS, Roe SM, Barford D. "Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex". Nature 434(7033):663-666 (2005).

Parlato, R., and Kreiner, G. "Nucleolar activity in neurodegenerative diseases: a missing piece of the puzzle? " J Mol Med (Berl) 91, 541-547 (2013).

Parlato R, Liss B." How Parkinson's disease meets nucleolar stress". Biochim biophys Acta 1842(6):791-7 (2014).

Parry L and Clarke AR." The Roles of the Methyl-CpG Binding Proteins in Cancer". Genes Cancer 2(6):618-30 (2011).

Patil NA, Gade WN, Deobagkar DD." Epigenetic modulation upon exposure of lung fibroblasts to TiO2 and ZnO nanoparticles: alterations in DNA methylation". Int J nanomedicine 11:4509-4519 (2016).

Pena E, Berciano MT, Fernandez R, Ojeda JL, Lafarga M. "Neuronal body size correlates with the number of nucleoli and Cajal bodies, and with the organization of the splicing machinery in rat trigeminal ganglion neurons". J Comp Neurol; 430:250–63. (2001).

Penas C and Navarro X."Epigenetic modifications associated to neuroinflammation and neuropathic pain after neural trauma". Front Cell Neurosci 7; 12:158 (2018).

Peng Y, Yu S, Li H, Xiang H, Peng J, Jiang S. "MicroRNAs: emerging roles in adipogenesis and obesity". Cell Signal. 26(9):1888-96 (2014).

Peng C, Li L, Zhang MD, Bengtsson Gonzales C, Parisien M, Belfer I, Usoskin D, Abdo H, Furlan A, Häring M, Lallemend F, Harkany T, Diatchenko L, Hökfelt T, Hjerling-Leffler J and Ernfors P. "miR-183 cluster scales mechanical pain sensitivity by regulating basal and neuropathic pain genes. Science 356 1168–1171 (2017).

Pereira MA, Tao L, Wang W, Li Y, Umar A, Steele VE, Lubet RA. "Modulation by celecoxib and difluoromethylornithine of the methylation of DNA and the estrogen receptor-alpha gene in rat colon tumors". Carcinogenesis 25(10):1917-23 (2004).

Perisic T, Zimmermann N, Kirmeier T, Asmus M, Tuorto F, Uhr M, Holsboer F, Rein T, Zschocke J". Valproate and amitriptyline exert common and divergent influences o n global and gene promoter-

specific chromatin modifications in rat primary astrocytes". Neuropsychopharmacology 35(3):792-805 (2010).

Peters A, Palay SL, Webster H deF. "The fine structure of the nervous system. Neurons and their supporting cells". New York: Oxford University Press (1991).

Piedade D, Azevedo-Pereira JM. "The Role of microRNAs in the Pathogenesis of Herpesvirus Infection". Viruses 8(6):156 (2016).

Pietrzak M, Rempala G, Nelson P, Zheng J, and Hetman M. "Epigenetic Silencing of Nucleolar rRNA Genes in Alzheimer's Disease".PLoS one 6(7) (2011).

Politz JC, Zhang F, Pederson T." MicroRNA-206 colocalizes with ribosome-rich regions in both the nucleolus and cytoplasm of rat myogenic cells". Proc Natl Acad Sci USA 12; 103(50):18957-62 (2007).

Politz JC, Hogan EM, Pederson T." MicroRNAs with a nucleolar location". RNA 15(9) (2009).

Pollema-Mays S, Centeno M, Apkarian A and Martina M. "Expression of DNA methyltransferases in adult dorsal root ganglia is cell-type specific and up regulated in a rodent model of neuropathic pain". Front Cell Neurosciences, 8:217(2014).

Pradillo M, Santos JL." Genes involved in miRNA biogenesis affect meiosis and fertility". Chromosome Res (2018).

Price D. "Psychological and Neural Mechanisms of the Affective Dimension of Pain". Science 288: 1769-1772 (2000).

Qabaja A, Alshalalfa M, Bismar TA, Alhajj R. "Protein networkbased Lasso regression model for the construction of diseasemiRNA functional interactions". EURASIP Journal on Bioinformatics y Systems Biology (1):3 (2013).

Rahn EJ, Guzman-Karlsson MC and David Sweatt J." Cellular, molecular, and epigenetic mechanisms in non-associative conditioning: implications for pain and memory". Neurobiol Learn Mem 105:133-50 (2013).

Raska I, Shaw PJ, Cmarko D.” New insights into nucleolar architecture and activity”. Int Rev Cytol 255: 177 (2006).

Rathmell and Fields. “Harrison’s principles of internal medicine. Chapter 10: Pain: Pathophysiology and management”. 20th Edition (2015).

Rauch TA, Pfeifer GP.” The MIRA method for DNA methylation analysis”. Methods Mol biol 507:65-75 (2009).

Raut SK, Singh GB, Rastogi B, Saikia UN, Mittal A, Dogra N, Singh S, Prasad R, Khullar M. “miR-30c and miR-181a synergistically modulate p53-p21 pathway in diabetes induced cardiac hypertrophy”. Mol Cell Biochem. 417(1- 2):191-203(2016).

Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, Jenuwein T.” Regulation of chromatin structure by site-specific histone H3 methyltransferases”. Nature 10; 406(6796):593-9 (2000).

Reed ER, Latourelle JC, Bockholt JH, Bregu J, Smock J, Paulsen JS and Myers RH. “MicroRNAs in CSF as prodromal biomarkers for Huntington disease in the PREDICT-HD study”. Neurology 90(4) (2018).

Reik W, Dean W.” DNA methylation and mammalian epigenetics”. Electrophoresis 22(14):2838-43 (2001).

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. “The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans”. Nature 403(6772):901-906 (2000).

Ren W, Gao L and Song J. “Structural Basis of DNMT1 and DNMT3A-Mediated DNA Methylation”. Genes 11; 9(12) (2018).

Rhead B, Brorson IS, Berge T, Adams C, Quach H, Moen SM, Berg-Hansen P, Celius EG, Sangurdekar DP, Bronson PG, Lea RA, Burnard S, Maltby VE, Scott RJ, Lechner-Scott J, Harbo HF, Bos SD, Barcellos LF.” Increased DNA methylation of SLFN12 in CD4+ and CD8+ T cells from multiple sclerosis patients”.Plos One 31; 13(10) (2018).

Riancho J, Ruiz-Soto M , Villagrá N, Berciano J, Berciano MT and Lafarga M.” Compensatory Motor Neuron Response to Chromatolysis in the Murine hSOD1G93A Model of Amyotrophic Lateral Sclerosis”. Front Cell Neurosci 8;346 (2014).

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. “Identification of mammalian microRNA host genes and transcription units”. Genome Research 14(10A):1902-1910 (2004).

Rose NR and Klose RJ.” Understanding the relationship between DNA methylation and histone lysine methylation”. Biochim Biophys Acta 1839(2):1367-72 (2014).

Roubroeks JAY, Smith RG, van den Hove DLA, Lunnon K.” Epigenetics and DNA methylomic profiling in Alzheimer's disease and other neurodegenerative diseases”. J Neurochem 143(2):158-170 (2017).

Rupaimoole R and Slack FJ.” MicroRNA therapeutics: towards a new era for the management of cancer and other diseases”. Nat Rev Drug Discov, 16(3): 203-222 (2017).

Sado T, Fenner MH, Tan SS, Tam P, Shioda T, Li E. “X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation”. Dev Biol 15; 225(2):294-303 (2000).

Saito Y and Jones PA. “Epigenetic activation of tumor suppressor microRNAs in human cancer cells”. Cell cycle 5 (19) (2006).

Saito Y, Suzuki H, Imaeda H, Matsuzaki J, Hirata K, Tsugawa H, Hibino S, Kanai Y, Saito H, Hibi T.” The tumor suppressor microRNA-29c is downregulated and restored by celecoxib in humangastric cancer cells”. Int J Cancer 15; 132(8):1751-60 (2013).

Sakai A, Saitow F, Miyake N, Miyake K, Shimada T, Suzuki H. “Mir 7a alleviates the maintenance of neuropathic pain through regulation of neuronal excitability”. Brain a Journal of Neurology 136 (Pt 9):2738-2750 (2013).

Saksouk N, Simboeck E and Dejardin J. "Constitutive heterochromatin formation and transcription in mammals". Epigenetics Chromatin 8:3 (2015).

Salter MW1 and Beggs S2." Sublime microglia: expanding roles for the guardians of the CNS". Cell 3; 158(1):15-24 (2014)

Sanna MD, Guandalini L, Romanelli MN, Galeotti N. "The new HDAC1 inhibitor LG325 ameliorates neuropathic pain in a mouse model". Pharmacol Biochem Behav 160:70-75 (2017).

Sayed D and Abdellatif M." MicroRNAs in development and disease" Physiol Rev 91(3):827-887 (2011).

Schafferer S, Khurana R, Refolo V, Venezia S, Sturm E, Piatti P, Hechenberger C, Hackl H, Kessler R, Willi M, Gstir R, Krogsdam A, Lusser A, Poewe W, Wenning GK, Hüttenhofer A, Stefanova N." Changes in the miRNA-mRNA Regulatory Network Precede Motor Symptoms in a Mouse Model of Multiple System Atrophy: Clinical Implications". PLoS One 10; 11(3) (2016).

Schmidt U, Herrmann L, Hagl K, Novak B, Huber C, Holsboer F, Wotjak CT, Buell DR." Therapeutic Action of Fluoxetine is Associated with a Reduction in Prefrontal Cortical miR-1971Expression Levels in a Mouse Model of Posttraumatic Stress Disorder". Front Psychiatry 10; 4:66 (2013).

Scholz J, Woolf CJ. "The neuropathic pain triad: neurons, immune cells and glia". Nature Neuroscience 10(11):1361-1368 (2007).

Selamat SA, Galler JS, Joshi AD, Fyfe MN, Campan M, Siegmund KD, Kerr KM, Laird-Offringa IA." DNA methylation changes in atypical adenomatous hyperplasia, adenocarcinoma in situ, and lung adenocarcinoma". PLoS One 6(6) (2011).

Shao J, Cao J, Wang J, Ren X, Su S, Li M, Li Z, Zhao Q, Zang W." MicroRNA-30b regulates expression of the sodium channel Nav1.7 in nerve injury-induced neuropathic pain in the rat". Mol Pain 19; 12 (2016).

Shao C, Gao Y, Jin D, Xu X, Tan Shuying, Yu H, Zhao Q, Zhao L, Wang W and Wang D. "DNMT3a methylation in neuropathic pain". Journal of Pain Research.10 2253-2262 (2017).

Sharma G, Upadhyay S, Srilalitha M, Nandicoori VK, Khosla S." The interaction of mycobacterial protein Rv2966c with host chromatin is mediated through non-CpG methylation and histone H3/H4 binding". Nucleic Acids Res 30; 43(8):39222-37 (2015).

Shav-Tal Y, Blechman J, Darzacq X, Montagna C, Dye BT, Patton JG, Singer RH, Zipori D." Dynamic sorting of nuclear components into distinct nucleolar caps during transcriptional inhibition". Mol Biol Cell 16(5):2395-413 (2005).

Shen R, Tao L, Xu Y, Chang S, Van Brocklyn J, Gao JX." Reversibility of aberrant global DNA and estrogen receptor-alpha gene methylation distinguishescolorectal precancer from cancer ". Int J Clin Exp Pathol 2(1):21-33 (2009).

Shirai A, Kawaguchi T, Shimojo H, Muramatsu D, Ishida-Yonetani M, Nishimura Y, Kimura H, Nakayama JI and Shinkai Y." Impact of nucleic acid and methylated H3K9 binding activities of Suv39h1 on its heterochromatin assembly". Elife (2017).

Siddiqui ZH, Abbas ZK, Ansari MW and Khan MN." The role of miRNA in somatic embryogenesis". Genomics (2018).

Siedlecki P and Zielenkiewicz P." Mammalian DNA methyltransferases". Acta Biochim 53(2):245-56 (2006).

Siegmund KD, Connor CM, Campan M, Long TI, Weisenberger DJ, Biniszkiewicz D, Jaenisch R, Laird PW, Akbarian S" DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons". Plos One 19; 2(9) (2007).

Sirri V, Urcuqui-Inchima S, Roussel P, Hernandez-Verdun D." Nucleolus: the fascinating nuclear body". Histochem Cell Biol 129(1):13-31 (2008).

Skaper SD, Giusti P and Facci L. "Microglia and mast cells: two tracks on the road to neuroinflammation". Federation of American Societies for Experimental Biology Journal. 26(8):3103-3117 (2012).

Skinner CM, Ivanov NS, Barr SA, Chen Y, Skalsky RL." An Epstein-Barr Virus MicroRNA Blocks Interleukin-1 (IL-1) Signaling by Targeting IL-1 Receptor 1". J Virol 13; 91(21) (2017).

Smalheiser NR and Torvik VI. "Mammalian microRNAs derived from genomic repeats". Trends in Genetics 21(6):322-326(2005).

Smirnov E, Hornacek M, Kovacik L, Mazel T, Schrofel A, Svidenska S, Skalnikova M, Bartova E, Cmarko D and Raska I."Reproduction of the FC/DFC units in nucleoli". Nucleus 7(2):203-215 (2016).

Somasundaram S, Forrest M, Moinova H, Cohen A, Varadan V , LaFramboise T, Markowitz S, Khalil A." The DNMT1-associated lincRNA DACOR1 reprograms genome-wide DNA methylation in colon cancer". Clin Epigenetics 10; 127 (2018).

Song JJ, Liu J, Tolia NH, Schneiderman J, Smith SK, Martienssen RA, Hannon GJ, and Joshua-Tor L. "The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes". Nature Structural Biology 10(12):1026-1032 (2003).

Spector DL and Lamond AI." Nuclear speckles". Cold Spring Harb Perspect Biol, 1; 3 (2) (2011).

Stappert L, Roese-Koerner B, Brüstle O." The role of microRNAs in human neural stem cells, neuronal differentiation and subtype specification". Cell Tissue Res 359(1):47-64 (2015).

Steiman-Shimony A, 9Shtrikman O and Margalit H. "Assessing the functional association of intronic miRNAs with their host genes". RNA 24 (8):991-1004 (2018).

Stoica BA and Faden AI." Cell death mechanisms and modulation in traumatic brain injury". Neurotherapeutics, 7(1):3-12 (2010).

Störchel PH, Thümmler J, Siegel G, Aksoy-Aksel A, Zampa F, Sumer S, Schratt G.” A large-scale functional screen identifies Nova1 and Ncoa3 as regulators of neuronal miRNA function”. EMBO J 2; 34(17):2237-54 (2015).

Strickland ER, Woller SA, Garraway SM, Hook MA, Grau JW, Miranda RC.” Regulatory effects of intermittent noxious stimulation on spinal cord injury-sensitive microRNAs and their presumptive targets following spinal cord contusion”. Front Neural circuits 18; 8:117 (2014).

Sullivan LL, Chew K and Sullivan BA.” α satellite DNA variation and function of the human centromere”. Nucleus 4; 8(4):331-339 (2017).

Sun H, Maze I, Dietz DM, Scobie KN, Kennedy PJ, Damez-Werno D, Neve RL, Zachariou V, Shen L, Nestler EJ.” Morphine epigenomically regulates behavior through alterations in his tone H3 lysine 9dimethylation in the nucleus accumbens”. J Neurosci 28; 32(48):17454-64 (2012).

Sun Y, Sahbaie P, Liang Y, Li WW, Li XQ and Clark JD. “Epigenetic regulation of spinal CXCR2 signaling in incisional hypersensitivity in mice”. Anesthesiology, 1195 (5) (2013).

Sun T, Li W, Ling S. “miR-30c and semaphorin 3A determine adult neurogenesis by regulating proliferation and differentiation of stem cells in the subventricular zones of mouse”. Cell Prolif. 49(3):270-80 (2016).

Sun L, Zhao JY, Gu X, Liang L, Wu S, Mo K, Feng J, Guo W, Zhang J, Bekker A, Zhao X, Nestler EJ and Tao YX”. Nerve injury-induced epigenetic silencing of opioid receptors controlled by DNMT3a in primary afferent neurons”. Pain 158(6):637-648 (2017).

Sun C, Zhao C, Li S, Wang J, Zhou Q, Sun J, Ding Q, Liu M, Ding G.” EZH2 Expression is increased in BAP1-mutant renal clear cell carcinoma and is related to poor prognosis”. J Cancer 1; 9(20):3787-3796 (2018).

Sung HY, Choi EN, Ahn Jo S, Oh S, Ahn JH.” Amyloid protein-mediated differential DNA methylation status regulates gene expression in Alzheimer's disease model cell line”. Biochem Biophys Res Commun 4; 414 (4):700-5 (2011).

Svitich OA, Sobolev VV, Gankovskaya LV, Zhigalkina PV, Zverev VV.” The role of regulatory RNAs (miRNAs) in asthma”. Allergol Immunopathol 46(2):201-205 (2018).

Swygert SG, Peterson CL. “Chromatin dynamics: interplay between remodeling enzymes and histone modifications”. Biochim Biophys Acta 1839(8):728-36 (2014).

Syring I, Bartels J, Holdenrieder S, Kristiansen G, Müller SC, Ellinger J.” Circulating serum miRNA (miR-367-3p, miR-371a-3p, miR-372-3p and miR-373-3p) as biomarkers in patients with testicular germ cell cancer”. J Urol 193(1):331- (2015).

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A.” Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1”. Science 15; 324(5929):930-5 (2009).

Tan PH, Pao YY, Cheng JK, Hung KC, Liu CC. “MicroRNAbased therapy in pain medicine: Current progress and future prospects”. Acta Anaesthesiology Taiwan 51(4):171-176 (2013).

Tanic M, Yanowsky K, Rodriguez-Antona C, Andrés R, Márquez-Rodas I, Osorio A, Benítez J, Martinez-Delgado B. “Deregulated miRNAs in Hereditary Breast Cancer Revealed a Role for miR-30c in Regulating KRAS Oncogene”. 2012; 7(6):e38847 (2012).

Tapia O, Bengoechea R, Palanca A, Arteaga R, Val-Bernal JF, Tizzano EF, Berciano MT, Lafarga M.” Reorganization of Cajal bodies and nucleolar targeting of coilin in motor neurons of type I spinal muscular atrophy”. Histochem Cell Biol 137 (5):657-67 (2012).

Tapia O, Narcís JO, Riancho J, Tarabal O,Piedrafita L, Calderó J, Berciano MT, Lafarga M.” Cellular bases of the RNA metabolism dysfunction in motor neurons of a murine model of spinal muscular atrophy: Role of Cajal bodies and the nucleolus”. Neurobiol Dis 108:83-99 (2017).

Taves S, Berta T, Chen G and Ji RR. “Microglia and spinal cord synaptic plasticity in persistent pain”. Hindawi Neural Plasticity 2013:id753656 (2013).

Thomas B, Matson S, Chopra V, Sun L, Sharma S, Hersch S, Rosas H, Scherzer C, Ferrante R, and Matson W. “A novel method for detecting 7-methyl guanine reveals aberrant methylation levels in Huntingtondisease”. Anal Biochem 15; 436(2):112-120 (2013).

Thomson D, Bracken C and Goodall G. “Experimental strategies for microRNA target identification”. Nucleic Acids Research. 39(16):6845-6853 (2011).

Tigchelaar S, Streijger F, Sinha S, Flibotte S, Manouchehri N, So K, Shortt K, Okon E, Rizzuto MA, Malenica I, Courtright-Lim A, Eisen A, Keuren-Jensen KV, Nislow C, Kwon BK.” Serum MicroRNAs Reflect Injury Severity in a Large Animal Model of Thoracic Spinal Cord Injury”. Sci Rep; 7(1):1376 (2017)

Tochiki KK, Cunningham J, Hunt SP and Géranton SM.” The expression of spinal methyl-CpG-binding protein 2, DNA methyltransferases and histone deacetylases is modulated in persistent pain states”. Molecular Pain 27; 8:14 (2012).

Todd A and Koerber H.”Neuroanatomical substrates of spinal nociception”. In: McMahon SB, Koltzenburg M, editors. Wall and Melzack's Textbook of Pain. 5th edn. Edinburgh: Churchill Livingstone; 73–90 (2006).

Torres I and Fujimori D. “Functional coupling between writers, erasers and readers of histone and DNA methylation”. Curr Opin Struct Biol, 35:68-75 (2015).

Torvik, A. “Central chromatolysis and the axon reaction -- A reappraisal, Neuropath”. Appt. Neurobiol., 2: 423-432 (1976).

Tosolini M, Brochard V, Adenot P, Chebrout M, Grillo G, Navia V, Beaujean N, Francastel C, Bonnet-Garnier A, Jouneau A.” Contrasting epigenetic states of heterochromatin in the different types of mouse pluripotent stem cells”. Sci Reo 10; 8(1):5776 (2018).

Tramullas M, Lantero A, Díaz A, Morchón N, Merino D, Villar A, Buscher D, Merino R, Hurlé JM, Izpisúa-Belmonte JC, Hurlé MA. “BAMBI (bone morphogenetic protein and activin membrane-bound inhibitor) reveals the involvement of the transforming growth factor-beta family in pain modulation”. J Neurosci 27; 30(4):1502-11 (2010).

Tramullas M, Francés R, De la Fuente R, Velategui S, Carcelén M, García R, LLorca J and Hurlé MA. “MicroRNA-30c-5p modulates neuropathic pain in rodents”. Science Translational Medicine, 10 (2018).

Trebicka J, Anadol E, Elfimova N, Strack I, Roggendorf M, Viazov S, Wedemeyer I, Drebber U, Rockstroh J, Sauerbruch T, Dienes HP and Odenthal M.” Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis”. J Hepatol, 58(2); 234-9 (2013).

Trinkle-Mulcahy L and Sleeman J. “The Cajal body and the nucleolus: “In a relationship” or “It's complicated”?”RNA Biol, 14(6):739-751 (2017).

Tsai RY, Shen CH, Feng YP, Chien CC, Lee SO, Tsai WY, Lin YS, Wong CS.” Ultra-low-dose naloxone enhances the antinociceptive effect of morphine in PTX-treated rats: regulation on global histone methylation”. Acta Anaesthesiol Taiwan 50(3):106-11 (2012).

Tsoi H, Chan HY.” Expression of expanded CAG transcripts triggers nucleolar stress in Huntington's disease”. Cerebellum 12(3):310-2 (2013).

Tsoi H, Chan HY.” Roles of the nucleolus in the CAG RNA-mediated toxicity”. Biochim Biophys Acta 1842(6):779-84 (2014).

Turchinovich A, Tonevitsky AG, Burwinkel B.” Extracellular miRNA: A Collision of Two Paradigms”. Trends Biochem Sci 41(10):883-892 (2016).

Turek-Plewa J, Jagodziński PP.” The role of mammalian DNA methyltransferases in the regulation of gene expression”. Cell Mol Biol 10(4):631-47 (2005).

Turner BM.” Defining an epigenetic code”. Nat Cell Biol 9(1):2-6 (2007).

Tsuda M.” Microglia in the spinal cord and neuropathic pain”. J Diabetes Investig 7(1):17-26 (2016).

Vacante F, Denby L, Sluimer JC and Baker AH. “The function of miR-143, miR-145 and the MiR-143 host gene in cardiovascular development and disease”. Vascul Pharmacol (18) (2018).

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO.” Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells”. Nat Cell Biol 9(6):654-9 (2007).

Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. “Control of translation and mRNA degradation by miRNAs and siRNAs”. Genes and Development 20(5):515-524 (2006).

van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N.” Neuropathic pain in the general population: a systematic review of epidemiological studies”. Pain 155(4):654-62 (2014).

van Holde K and Zlatanova J.” Chromatin higher order structure: chasing a mirage?”. J Biol Chem 14; 270(15):8373-6 (1995).

Van Rooij E, Pucell A and Levin A. “Developing microRNA therapeutics”. Circ Res.110: 496-507 (2012).

Vasudevan S, Tong Y, Steitz JA.” Switching from repression to activation: microRNAs can up-regulate translation”. 23; 128(6):1105-18 (2007).

Verdu B, Decosterd I, Buclin T, Stiefel F, Berney A.” Antidepressants for the treatment of chronic pain”. Drugs 68(18):2611-32 (2008).

Verheggen C, Le Panse S, Almouzni G, Hernandez-Verdun D.” Maintenance of nucleolar machineries and pre-rRNAs in remnant nucleolus of erythrocyte nuclei and remodeling in Xenopus egg extracts”. Exp Cell Res 10; 269 (1): 23-34 (2001).

Vignier N, Amor F, Fogel P, Duvallet A, Poupiot J, Charrier S, Arock M, Montus M, Nelson I, Richard I, Carrier L, Servais L, Voit T, Bonne G, Israeli D.”

Distinctive serum miRNA profile in mouse models of striated muscular pathologies". PLoS One 8(2) (2013).

Villagra A, Sotomayor EM, Seto E." Histone deacetylases and the immunological network: implications in cancer and inflammation". Oncogene 14; 29(2):157-73 (2010).

Villar AV, Merino D, Wenner M, Llano M, Cobo M, Montalvo C, García R, Martín-Durán R, Hurlé JM, Hurlé MA, Nistal JF." Myocardial gene expression of microRNA-133a and myosin heavy and light chains, in conjunction with clinical parameters, predict regression of left ventricular hypertrophy after valve replacement in patients with aortic stenosis". Heart 97(14):1132-7 (2011).

Villar AV, García R, Merino D, Llano M, Cobo M, Montalvo C, Martín-Durán R, Hurlé MA, Nistal JF." Myocardial and circulating levels of microRNA-21 reflect left ventricular fibrosis in aortic stenosis patients". Int J Cardiol 10; 167(6):2875-81 (2013).

von Schack D, Agostino MJ, Murray BS, Li Y, Reddy PS, Chen J, Choe SE, Strassle BW, Li C, Bates B, Zhang L, Hu H, Kotnis S, Bingham B, Liu W, Whiteside GT, Samad TA, Kennedy JD, Ajit SK." Dynamic changes in the microRNA expression profile reveal multiple regulatory mechanisms in the spinal nerve ligation model of neuropathic pain". PLoS 14; 6(3) (2011).

Waddington CH."The epigenotype". Int J Epidemiol 41(1):10-3 (2012).

Wagner EK, Albaugh BN, Denu JM." High-throughput strategy to identify inhibitors of histone-binding domains". Methods Enzymol 512:161-85 (2012).

Walsh CP, Chaillet JR, Bestor TH. "Transcription of IAP endogenous retroviruses is constrained by cytosine methylation". Nat Genet. 20:116–117 (1998).

Walton EL, Francastel C, Velasco G." Dnmt3b Prefers Germ Line Genes and Centromeric Regions: Lessons from the ICF Syndrome and Cancer and Implications for Diseases". Biology 4; 3(3):578-605 (2014).

Wang Z. "MicroRNA: A matter of life or death". World Journal of Biological Chemistry 1(4):41-54 (2010).

Wang Y, Neumann M, Hansen K, Hong SM, Kim S, Noble-Haeusslein LJ, Liu J."
Fluoxetine increases hippocampal neurogenesis and induces epigeneti c factors but does not improve functional recovery after traumatic brain injury". J Neurotrauma 28(2):259-68 (2011).

Wang F, Stefano GB, Kream RM." Epigenetic modification of DRG neuronal gene expression subsequent to nerve injury: etiological contribution to complex regional pain syndromes (Part I)". Med Sci Monit 25; 20:1067-77 (2014).

Wang Y, Lin ZP, Zheng HZ, Zhang S, Zhang ZL, Chen Y, You YS, Yang MH." Abnormal DNA methylation in the lumbar spinal cord following chronic constriction injury in rats". Neurosci Lett 1; 610:1-5 (2016).

Watahiki A, Wang Y, Morris J, Dennis K, O'Dwyer H, Gleave M, Gout P, Wang Y." MicroRNAs Associated with Metastatic Prostate Cancer". PLoS One 6(9) (2011).

Watkin L, Mishra R, Gil A, Aslan N, Ghersi D,Luzuriaga K and Selin L. "Unique Influenza A crossreactive memory CD8 TCR repertoire has a potential to protect against Epstein Barr virus seroconversion". J Allergy Clin Immunol, 140(4) (2018).

Watson WE. "Cellular responses to axotomy and to related procedures" . Br Med Bull 30:112–115 (1974).

Watson CJ, Collier P, Tea I, Neary R, Watson JA, Robinson C, Phelan D, Ledwidge MT, McDonald KM, McCann A, Sharaf O, Baugh JA." Hypoxia-induced epigenetic modifications are associated with cardiac tissue fibrosis and the development of a myofibroblast-like phenotype". Hum Mol Genet 15; 23(8):2176-88 (2014).

Welin D, Novikova LN, Wiberg M, Kellerth JO, Novikov LN. "Survival and regeneration of cutaneous and muscular afferent neurons after

peripheral nerve injury in adult rats". Exp Brain Res 186(2):315-23 (2008).

Wen M, Men R, Liu X, Yang L. "Involvement of miR-30c in hepatic stellate cell activation through the repression of plasminogen activator inhibitor-1". Life Sci.155:21-8 (2016).

Wiffen PJ, Derry S, Moore RA, Kalso EA." Carbamazepine for chronic neuropathic pain and fibromyalgia in adult s". Cochrane DAtabase Syst Rev 10(4) (2014).

Wilczynski M, Danielska J, Domanska-Senderowska D, Dzieniecka M, Szymanska B, Malinowski A." Association of microRNA-200c expression levels with clinicopathological factors and prognosis in endometrioid endometrial cancer". Acta ObstetGynecol Scand, 97(5): 560-569 (2018).

Willemen HLDM, Huo XJ, Hao-Ying QL, Zijlstra J, Heijnen CJ and Kavelaars A. "MicroRNA-124 as a novel treatment for persistent hyperalgesic". Journal of Neuroinflammation 9:143 (2012).

Woodcock, C. L. F. J. Cell Biol. 59, 368a (1973).

Woolf CJ and Ma Q. "Nociceptors-noxious stimulus detectors". Neuron. 2; 55(3): 353-364 (2007).

Wong TY, Li F, Lin SM, Chan FL, Chen S, Leung LK." Celecoxib increases miR-222 while deterring aromatase-
expressing breast tumor growth in mice". BMC Cancer 12; 14:426 (2014).

Wu Ct, Morris JR." Genes, genetics, and epigenetics: a correspondence". Science 10; 293(5532):1103-5 (2001).

Wysocka J." Identifying novel proteins recognizing histone modifications using peptide pull-down assay". Methods 40(4):339-43 (2006).

Xing Y, Zheng X, Li G, Liao L, Cao W, Xing H, Shen T, Sun L, Yang B, Zhu D. "MicroRNA-30c contributes to the development of hypoxia pulmonary hypertension by inhibiting platelet-derived growth factor receptor β expression". Int J Biochem Cell Biol. 64:155-66 (2015).

Xu H, Pillai R, Azzouz T, Shpargel K, Kambach C, Hebert M, Schumperli D and Matera, A. "The C-terminal domain of coilin interacts with Sm proteins and U snRNPs". Chromosome. 114, 155-166 (2005)

Xu B, Cao J, Zhang J, Jia S, Wu S, Mo K, Wei G, Liang L, Miao X, Bekker A and Tao YX. "Role of MicroRNA-143 in Nerve Injury-Induced Upregulation of Dnmt3a Expression in Primary Sensory Neurons". Front Mol Neuroscience, 10:350 (2017).

Yadav R and Weng HR." EZH2 regulates spinal neuroinflammation in rats with neuropathic pain". Neuroscience 4; 349:106-117 (2017).

Yan KS, Yan S, Farooq A, Han A, Zeng L, Zhou MM. "Structure and conserved RNA binding of the PAZ domain". Nature 426(6965):468-474(2003).

Yang Q, Cui J, Wang P, Du X, Wang W, Zhang T, Chen Y. "Changes in interconnected pathways implicating microRNAs are associated with the activity of apocynin in attenuating myocardial fibrogenesis". Eur J Pharmacol. 784:22- 32 (2016).

Yang Z, He J, Gao P, Niu Y, Zhang J, Wang L, Liu M, Wei X, Liu C, Zhang C, Wang W, Du J, Li H, Hu W, Sun G." miR-769-5p suppressed cell proliferation, migration and invasion by targeting TGFBR1 in non-small cell lung carcinoma". Oncotarget 8; 8(69):113558-113570 (2017).

Yang Q, Li P, Wen Y, Li S, Chen J, Liu X, Wang L, Li X." Cadmium inhibits lysine acetylation and succinylation inducing testicular injury of mouse during development". Toxicol Lett 291; 112-120 (2018).

Yang Y, Liu Y, Li G, Li L, Geng P, Song H." microRNA-214 suppresses the growth of cervical cancer cells by targeting EZH2". Oncol Lett 16(5):5679-5686 (2018).

Yang K, Yang J and Yi J."Nucleolar Stress: hallmarks,sensing mechanisms and diseases". Cell Stress (2018).

Yeung K, Chung B, Choufani S, Mok M, Wong W, Mak C, Yang W, Lee P, Wong W, Chen Y, Grafodatskaya D, Wong R, Lau C, Chan D, Weksberg R, Yu-Lung Lau." Genome-Wide DNA Methylation Analysis of Chinese Patients with Systemic Lupus Erythematosus Identified

Hypomethylation in Genes Related to the Type I Interferon Pathway". Plos One 12(1) (2017).

Yi R, Qin Y, Macara IG, Cullen BR. "Exportin-5 mediates the nuclear export of premicroRNA and short hairpin RNAs". Genes y Development 17(24):3011-3016 (2003).

Yu F, Deng H, Yao H, Liu Q, Su F, Song E." Mir-30 reduction maintains self-renewal and inhibits apoptosis in breast tumor-initiating cells". Oncogene 22; 29 (29):4194-204 (2010).

Yu C, Chen WP, Wang XH. "MicroRNA in osteoarthritis". Journal of International Medical Research 39(1):1-9 (2011).

Yu Y, Scheel TKH, Luna JM, Chung H, Nishiuchi E, Scull MA, Echeverría N, Ricardo-Lax I, Kapoor A, Lipkin WI, Divers TJ, Antczak DF, Tennant BC, Rice CM." miRNA independent hepacivirus variants suggest a strong evolutionary pressure to maintain miR-122 dependence". PLoS Pathog 30; 13(10) (2017).

Yu QF, Liu P, Li ZY, Zhang CF, Chen SQ, Li ZH, Zhang GY, Li JC." MiR-103/107 induces tumorigenicity in bladder cancer cell by suppressing PTEN". Eur Rev Med Pharmacol Sci 22(24):8616-8623 (2018).

Yunta M, Nieto-Díaz M, Esteban FJ, Caballero-López M, Navarro-Ruíz R, Reigada D, Pita-Thomas DW, del Águila A, Muñoz-Galdeano T, Bibliografía 180 Maza RM. "MicroRNA dysregulation in the spinal cord following traumatic injury". PLOS ONE 7 (4):e34534 (2012).

Zadel M, Maver A, Kovanda A, and Peterlin B. "DNA Methylation Profiles in Whole Blood of Huntington's Disease Patients". Frontiers in Neurology 9; 655 (2018).

Zaiou M, El Amri H, Bakillah A." The clinical potential of adipogenesis and obesity-related microRNAs". Nutr Metab Cardiovasc Dis 28(2) _91-111 (2018).

Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. "Plasma microRNA profiling reveals loss of endothelial miR-126 and other

microRNAs in type 2 diabetes". Circulation Research 107(6):810-817 (2010).

Zeng Y. "Principles of micro-RNA production and maturation". Oncogene 25:6156–6162 (2006).

Zhang Z, Cai YQ, Zou F, Bie B, Pan ZZ." Epigenetic suppression of GAD65 expression mediates persistent pain". Nature medicine 9; 17(11):1488-55 (2011).

Zhang J, Banerjee B. "Role of MicroRNA in visceral pain" Journal Neurogastroenterology Motil 21(2):159-171 (2015).

Zhang R, Huang M, Cao Z, Qi J, Qiu Z, Chiang LY." MeCP2 plays an analgesic role in pain transmission through regulating CREB / miR-132 pathway". Mol Pain 12; 11:19 (2015).

Zhang J, Liang L, Miao X, Wu S, Cao J, Tao B, Mao Q, Mo K, Xiong M, Lutz BM, Bekker A, Tao YX." Contribution of the Suppressor of Variegation 3-
9 Homolog 1 in Dorsal Root Ganglia and SpinalCord Dorsal Horn to Ne rve Injury-induced Nociceptive Hypersensitivity". Anesthesiology 125(4):765-78 (2016).

Zhang Q, Huang C, Yang Q, Gao L, Liu HC, Tang J, Feng WH. "MicroRNA30c Modulates Type I IFN Responses To Facilitate Porcine Reproductive and Respiratory Syndrome Virus Infection by Targeting JAK1". J Immunol 196(5):2272-82(2016).

Zhang, Y., Chen, S. R., Laumet, G., Chen, H., and Pan, H. L. "Nerve injury diminishes opioid analgesia through lysine methyltransferase-mediated transcriptional repression of mu-opioid receptors in primary sensory neurons". J. Biol. Chem. 291, 8475–8485(2016).

Zhang Y, Cao N, Huang Y, Spencer CI, Fu JD, Yu C, Liu K, Nie B, Xu T, Li K, Xu S, Bruneau BG, Srivastava D, Ding S." Expandable Cardiovascular Progenitor
Cells Reprogrammed from Fibroblasts". Cell Stem Cell 3; 18(3):368-81 (2016).

Zhang C, Zhang G and Liu D.” Histone deacetylase inhibitors reactivate silenced transgene in vivo”. Gen Ther (2018).

Zhao J, J, Yu-Tzu Tai, Treon S, Pinkus G , Hideshima T, Bouxsein M, Munshi N, Anderson K and Carrasco R. “miR-30-5p functions as a tumor suppressor and novel therapeutic tool by targeting the oncogenic Wnt/β-catenin/BCL9 pathway”. Cancer Res 74(6):1801-1813 (2014).

Zhao JY, Liang L, Gu X, Li Z, Wu S, Sun L, Atianjoh FE, Feng J, Mo K, Jia S, Lutz BM, Bekker A, and Tao YX. “DNA methyltransferase DNMT3a contributes to neuropathic pain by repressing Kcna2 in primary afferent neurons”. Nature Communications, 8; 8 (2017).

Zhao H, Sun P, Fan T, Yang X, Zheng T and Sun C.” The effect of glutamate-induced excitotoxicity on DNA methylation in astrocytes in new in vitroneuron-astrocyte-endothelium co-culture system”. Biochem Biophys Res Commun (2018).

Zhe Fan, Huirong Jing, Jihong Yao, Yang Li, Xiaowei Hu, Huizhu Shao, Gang Shen, Jiyong Pan, Fuwen Luo, Xiaofeng Tian.” The Protective Effects of Curcumin on Experimental Acute Liver Lesion Induced by Intestinal Ischemia-Reperfusion through Inhibiting the Pathway of NF-κB in a Rat Model”. Oxid Med Cell Longev (2014).

Zheng N, Wang L, Hou Y, Zhou X, He Y, Wang Z.” Rottlerin inhibits cell growth and invasion via down-regulation of EZH2 in prostate cancer”. Cell cycle 17(21-22):2460-2473 (2018).

Zhou Y, Zheng X, Chen LJ, Xu B, Jiang JT.” microRNA-181b suppresses the metastasis of lung cancer cells by targeting sex determining region Y-related high mobility group-box 6 (Sox6)”. Pathol Res Pract (2018).

Zhou XL, Yu LN, Wang Y, Tang LH, Peng YN, Cao JL, Yan M.” Increased methylation of the MOR gene proximal promoter in primary sensory neurons plays a crucial role in the decreased analgesic effect of opioids in neuropathic pain”. Mol Pain 13; 10:51 (2014).

Zilberman D.” An evolutionary case for functional gene body methylation in plants and animals”. Genome Biol 9; 18(1):87 (2017).

Zimmermann M. "Ethical Guidelines for investigations of experimental pain in conscious animals". Pain, 16, 109-110 (1983).

Zippo A, Serafini R, Rocchigiani M, Pennacchini S, Krepelova A, Oliviero S." Histone crosstalk between H3S10ph and H4K16ac generates a histone code that mediates transcription elongation". Cell 18; 138(6); 1122-36 (2009).