Effects of genetic manipulation of the monoaminergic system on chronic pain and on the affective disorders associated with pain

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

Neuropathic pain is a pathologic and chronic condition that results from damage or dysfunction of the peripheral or central nervous system causing spontaneous pain and a pathologically amplified response to both noxious and non-noxious stimuli. This condition is very afflictive, affecting 7-8% of adult individuals worldwide. The noradrenergic system is of high importance in the neuronal interactions responsible for pain modulation and is also involved in the regulation of emotional/affective behaviours in response to pain.

The Locus Coeruleus (LC) is the main regulator of noradrenergic functions in the central nervous system and is the main source of noradrenergic innervation to the spinal dorsal horn. Besides projecting to the spinal dorsal horn, the LC also projects to many supraspinal brain regions, in particular to pre-frontal cortex areas, and plays a pivotal role in diverse behaviors such as learning, strategic behavior, motivation, and arousal. Furthermore, LC-derived noradrenaline is also causally linked to aversive states like stress, anxiety and depression.

The main goal of this project was to study the effects of selectively knocking-down at the LC the production of Dbh, the rate-limiting enzyme of noradrenaline synthesis, on the induction and development of neuropathic pain and of associated affective disorders, such as anxiety and depression behaviors. This was done by using mutant mice and the Cre-Lox system which allowed to spatially and temporally manipulate the noradrenergic system. For this purpose, mice with a Dbh flox$^{+/+}$ genotype were used to establish a protocol for *Dbh* gene ablation by stereotaxic injection of a lentivirus expressing the Cre recombinase enzyme (LV-Cre), into the LC. The mice underwent then a spared nerve injury (SNI) surgery to induce a well-established model of neuropathic pain (SNI model). The tests to evaluate anxiety- and depression-like behaviors showed similar results in neuropathic pain (SNI) and sham animals. Thus, mice showed a similar exploratory behavior in the open field, as well as a comparable behavior in the Elevated Plus Maze, but with tendency to spend more time in the closed arms than in open arms. On the Marble Burying test, the SNI group buried a few higher number of marbles, while in the forced swimming test, the only one that evaluates depressive-like behaviors, no significant differences were observed. The nociceptive tests confirmed the hypersensitivity typical of a neuropathic pain condition. After stereotaxic injection of LV-Cre, a heterogeneous Dbh ablation was detected in different rostro-caudal areas of the LC. At the behavioral level the data showed different effects on the nociceptive and anxiety and depressive-like behaviors according to the rostro-caudal level of the LC targeted by LV-Cre injection. This suggests that different rostro-caudal regions of the LC

might be differentially engaged in the processing of the nociceptive and affective components of pain.

Keywords: Pain; Emotion; Monoamine; Anxiety; Depression; Locus Coeruleus

Resumo

A dor neuropática é uma condição patológica e crónica que resulta de dano ou disfunção do sistema nervoso central ou periférico, causando dor espontânea e uma resposta patologicamente amplificada a estímulos nóxicos e não nóxicos. Esta condição é muito aflitiva, afetando 7 a 8% das pessoas adultas em todo o mundo. O sistema noradrenérgico é de grande importância nas interações neuronais responsáveis pela modulação da dor e também está envolvido na regulação dos comportamentos emocionais / afetivos em resposta à dor.

O Locus Coeruleus (LC) é o principal regulador das funções noradrenérgicas no sistema nervoso central e é a principal fonte de inervação noradrenérgica para o corno dorsal da medula espinhal. Além de projetar para o corno dorsal da medula espinhal, o LC projeta também para muitas regiões encefálicas supraespinhais, em particular para as áreas do córtex pré-frontal, e desempenha um papel fundamental em comportamentos diversos, como aprendizagem, comportamento estratégico, motivação e atenção focalizada. Além disso, a noradrenalina derivada do LC também está causalmente associada a estados aversivos, tais como ansiedade e depressão.

O principal objetivo deste projeto foi estudar os efeitos de deletar seletivamente no LC a produção de Dbh, a enzima limitante da síntese da noradrenalina, na indução e desenvolvimento de dor neuropática e de distúrbios afetivos associados, tais como comportamentos de ansiedade e depressão. Isto foi feito através da utilização de ratinhos mutantes e do sistema Cre-Lox que permitiram a manipulação espacial e temporal do sistema noradrenérgico. Para este fim, os ratinhos com o genótipo Dbh flox$^{+/+}$ foram utilizados para estabelecer um protocolo para ablação do gene *Dbh* por injeção estereotáxica de, um lentivírus que expressa a enzima Cre recombinase (LV-Cre), no LC. Os ratinhos foram sujeitos depois a uma cirurgia de lesão do nervo ciático para induzir um modelo bem estabelecido de dor neuropática (modelo SNI). Os testes comportamentais para ansiedade e depressão apresentaram resultados semelhantes em animais com dor neuropática (SNI) e sham. Desta forma foi observado um comportamento exploratório em campo aberto semelhante, bem como um comportamento comparável no labirinto Elevated Plus, mas com tendência para permanecerem mais tempo nos braços fechados do que nos braços abertos. No teste dos berlindes, o grupo SNI enterrou um número maior de berlindes, enquanto no teste de natação forçada, o único que avalia o comportamento depressivo, não foram observadas diferenças significativas. Os testes nociceptivos confirmaram a hipersensibilidade típica de uma condição de dor neuropática. Após a injeção estereotáxica das partículas lentivíricas de LV-Cre no LC, uma ablação heterogénea de

Dbh foi detetada em diferentes áreas rostro-caudais do LC. Ao nível comportamental, os dados mostraram diferentes efeitos nos comportamentos nociceptivos e de ansiedade e depressão, de acordo com o nível rostro-caudal do LC alvejado pela injeção de LV-Cre. Isto sugere que diferentes regiões rostro-caudais do LC poderão estar envolvidas de forma diferencial no processamento dos componentes nociceptivos e afetivos da dor.

Abbreviatures

AAV	Adeno-associated virus
ACC	Anterior Cingulate Cortex
AMY	Amygdala
AP	Anteroposterior
AR	Adrenergic receptors
ASICs	Acid-sensing ion channels
AV	Adeno Virus
BA	Basolateral Amygdala
BLA	Basolateral complex
CCI	Chronic Constriction Injury
CeA	Central Nucleus of Amygdala
CKO	Conditional Knockout
CNS	Central Nervous System
COMT	Catecol O-Metiltransferase
DAB	Diaminobenzidine
DBH	Dopamine-β-hydroxylase
DLF	Dorsolateral Funiculus
DRG	Dorsal Root Ganglia
DRt	Dorsal Reticular Nucleus
DV	Dorsoventral
EGFP	Enhanced Green Fluorescent Protein
EPM	Elevated Plus Maze
ERK1/2	Extracellular-signal regulated kinases ½
FST	Forced Swimming Test
GFP	Green Fluorescent Protein
HFV	Human Foamy Virus
HSV	Herpes simplex Virus
HSV-1	Herpes Simplex Virus-1
IASP	Association for the Study of Pain
IC	Insular Cortex
LA	Lateral Nucleus of Amygdala
LC	Locus Coeruleus
LM	Lateromedial
MAO	Monoamine Oxidase
MB	Marble Burying
NA	Noradrenaline

NAc	Nucleus Accumbens
NGS	Normal Goat Serum
NRM	Nucleus Raphe Magnus
OF	Open Field
PAG	Periaqueductal Gray Region
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline-Triton
PFC	Prefrontal Cortex
PSL	Partial Sciatic Ligation
rACC	Rostral Anterior Cingular Cortex
RVM	Rostral Ventromedial Medulla
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
SNI	Spared Nerve Injury
SNL	Spinal Nerve Ligation
TCAs	Tricyclic Antidepressants
TG	Trigeminal Ganglia
TH	Tyrosine Hydroxylase
TRP channels	Transient Receptor as Potential-generating channels
VTA	Ventral Tegmental Area

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I. Introduction

Pain is a serious condition and often debilitating for people who live with it, not only for the ones that are suffering with that, but also for their families and society in general[1]. However, pain is a common occurrence in the entire life, a survival mechanism that functions as an early warning sign of ongoing or impending tissue damage[1, 2]. Besides the association of painwith injury and disease, pain is a subjective symptom and it is that subjectivity the main challenge for current and further investigations.

1. Pain

Until the 1960s, pain was considered an inevitable sensory response to tissue damage without minimal considerations for the affective dimension of the pain experience and none for the effects of genetic differences, past experience, anxiety or expectation[3]. The newest advances have been made in our understanding of the mechanisms that underlie pain and in the treatment of people who complain of pain[3, 4].

The Melzack-Wall gate control theory, proposed in 1965, demonstrated the mechanisms in the central nervous system that control the perception of the noxious stimulus, and thus integrated afferent, upstream processes, with downstream modulation from the brain [3-5]. The gate control theory proposed that the transmission of nerve impulses from afferent fibers to spinal cord transmission cells is modulated by a gating mechanism in the spinal dorsal horn[6]. This theory considers the motivational dimension of pain in addition to its more obvious sensory dimension[7]. Considering this, the painful sensation was defined as being comprised by three different components, a sensory-discriminative component that refers to the capacity to analyze the nature, location, intensity, and duration of the nociceptive stimulation; an affective-motivational component that involves the unpleasant character of all the painful perception; and a cognitive-evaluative component related to the past experiences[8].

The most complete definition of pain has been provided by the International Association for the Study of Pain (IASP), which established pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”[9].

1.1. Types of pain

The existence of many types of pain can be understood by the identification of four broad categories: nociception, perception of pain, suffering, and pain behaviors[3]. Pain may be divided into several types according to its duration, etiology, localization or others, contributing to its complexity. Regarding the duration, pain is classified as transient, acute or chronic. Transient pain is elicited by the activation of nociceptive transducers in the skin or other tissues of the body in the absence of any tissue damage. Transient pain has evolved to protect man from physical damage by the environment or by over stress of the body tissues. This is present in every day of life and is rarely a reason to seek health care[3]. Acute pain is defined as a short duration, phasic and intense physiological event, which normally has a rapid resolution after the phenomenon that initially originated pain is healed. The majority of cases of acute pain is caused by substantial injury of body tissue with activation of nociceptive transducers at the local of the tissue damage. This injury alters the characteristics of special sensory fibers, called nociceptors, their central connections, and the autonomic nervous system in the region. Chronic pain, in contrast, is a long-lasting persistent pathological pain that is present even when there is no apparent biological cause, or the original cause is no longer present. Indeed, the original injury may be capable of damaging the nervous system in such way so that it is unable to restore itself to a normal state. Other chronic pain syndromes may occur spontaneously without any sign of injury. In all of them, the intensity of pain is out of proportion and leads to health care[3]. Chronic Pain can be classified into 3 categories: nociceptive pain, which arises from non-neural tissue damage; neuropathic pain in which there is nervous tissue damage; or a mix of the two categories. Nociceptive pain is the physiological sensation caused by the activation of neuronal pathways by stimuli with sufficient intensity to potentially cause tissue damage. Stimuli detection is named nociception and is a critical protective process that helps to prevent injury by generating a reflex withdrawal from stimuli associated to an unpleasant sensation, namely pain[10]. Neuropathic pain is difficult to treat being associated to spontaneous pain, exaggerated responses to nociceptive stimuli (hyperalgesia) and nociceptive responses to stimuli which are normally non-nociceptive (allodynia)[11].

1.2. Pain transmission

Pain transmission is initiated by the activation of specialized sensory fibers widely distributed throughout the body, called nociceptors. These are high-threshold sensory receptors of the peripheral nervous system capable of transducing mechanical, chemical and thermal noxious stimuli from different organism locations into action potentials. The cell bodies of these neurons are located at trigeminal ganglia (TG) in the cephalic region and at dorsal root ganglia (DRG) in extracephalic regions[12, 13]. Nociceptors' cell bodies possess a unique axon that bifurcates into two branches. A central branch projects to the dorsal horn of the spinal cord while the peripheral branch terminates in one of the many peripheral organs, and constitutes the sensitive fiber. There are three types of fibers, distinguished according to their diameter, myelination degree and conduction velocity (Aβ, Aδ and C)[13, 14].These include the two major classes of nociceptors. The medium diameter myelinated (Aδ) afferents mediate acute, well-localized "first" or fast pain. These fibers differ considerably from the larger diameter and rapidly conducting Aβ fibers that respond to innocuous mechanical stimulation applied to skin, muscle and joints and thus not contribute to pain. The second class of nociceptors includes the small diameter unmyelinated C fibers that convey poorly localized, "second" or slow pain[15, 16].
The physiochemical properties of noxious stimuli are converted to electrical activity by ion channels such as transient receptor potential-generating channels (TRP channels) and purinergic channels, and this electrical activity is amplified by sodium channels to elicit action potentials[17].
The nociceptive signal is transmitted at central synapses through release of a variety of neurotransmitters that have the potential to excite second-order nociceptive projection neurons in the spinal dorsal horn or hindbrain (Figure 1). These neurons project to supra-spinal sites, which further project to cortical and subcortical regions via third-order neurons, enabling the encoding and perception of the multidimensional pain experience.
The transmission of nociceptive signals at the spinal dorsal horn can be modulated by local inhibitory GABAergic and opioidergic interneurons and also by descending serotonergic and noradrenergic projections influencing the response to and perception of pain[18].

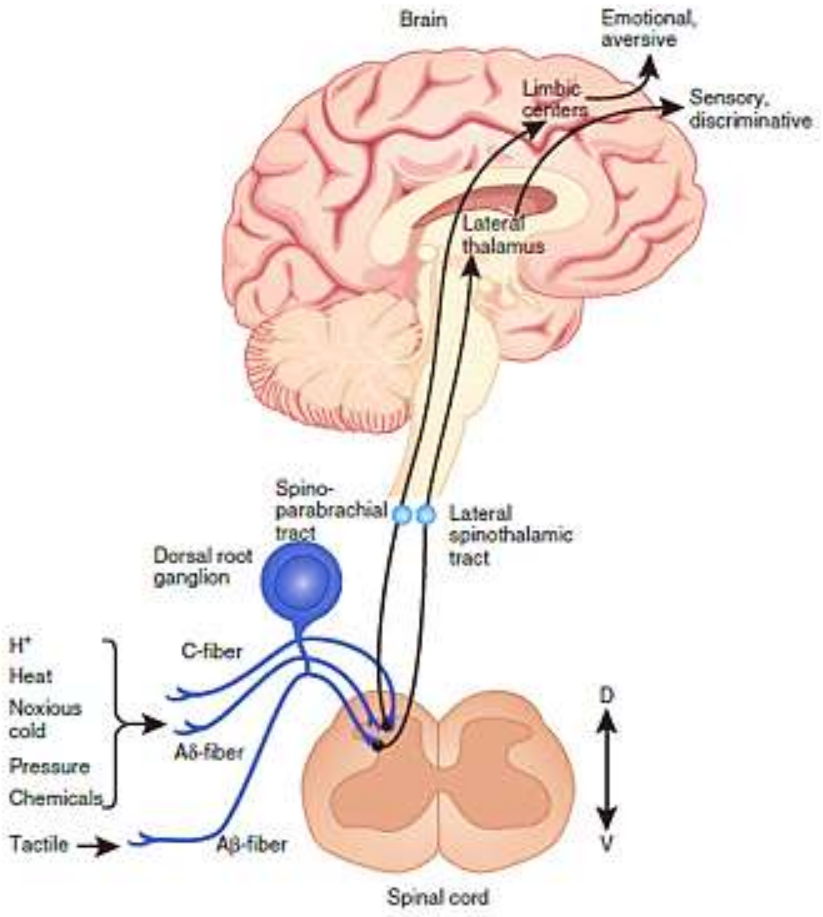


Figure 1- Pain circuits. Schematic overview of the main circuits mediating physiological pain. Nociceptive signals are transmitted from the periphery by nociceptive sensory neurons (first-order primary afferent neurons) the peripheral terminals of which are clustered with ion channels, including transient receptor potential channel subtypes (TRPA, TRPM and TRPV), sodium channel isoforms (Nav), potassium channel subtypes (KCNK) and acid-sensing ion channels (ASICs). The transduction of external noxious stimuli is initiated by membrane depolarization due to the activation of these ion channels. Action potentials are conducted along the axons of nociceptive Aβ- and C-fibers, through the cell body in the dorsal root ganglion to the axonal terminals, which form the presynaptic element of central synapses of the sensory pathway in the spinal dorsal horn or hindbrain. The central terminals of Aβ- and C-fibers synapse with interneurons and second-order nociceptive projection neurons, primarily within the superficial laminae of the spinal dorsal horn. The axons of second-order nociceptive projection neurons decussate at the spinal cord level, joining the ascending fibers of the anterolateral system, and project to brainstem and thalamic nuclei, transferring information about the intensity and duration of peripheral noxious stimuli. No single brain region is essential for pain, but rather pain results from the activation of a distributed group of structures. Third-order neurons from the thalamus project to several cortical and subcortical regions that encode sensory-discriminative, emotional and cognitive aspects of pain. Several brainstem sites are also known to contribute to the descending modulation of pain; (Adapted from Kunner et al[17]).

1.3. Pain modulation

1.3.1. The endogenous pain control system

Pain transmission at spinal cord can be modulated through a complex process known as endogenous pain modulation. Most of the ascending axons of spinal second order neurons project to the brainstem reticular formation, which includes areas in charge of pain modulation, such as dorsal reticula nucleus (DRt), the rostral ventromedial medulla (RVM), the noradrenergic locus coeruleus(LC) and the periaqueductal gray region (PAG)[12]. All of these supraspinal sites play an important role in pain modulation but, the most well characterized pain modulatory areas are the mesencephalic periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM) (Figure 2).

Pain modulation exists in the form of a descending pain modulatory circuit with inputs that arise from multiple areas, including the hypothalamus, the amygdala and the rostral anterior cingular cortex (rACC), feeding to the midbrain periaqueductal gray region (PAG), and with outputs from the PAG to the medulla oblongata[12, 19]. This modulatory process works to inhibit or facilitate the spinal nociceptive processing, which ultimately controls the experience of pain. The PAG, a key region for descending inhibition receives projections from regions associated with the processing of emotions and projects to the rostral ventromedial medulla (RVM), which includes the serotonin-rich nucleus raphe magnus (NRM) as well as the nucleus reticularis gigantocellularis pars alpha and the nucleus paragigantocellularis lateralis[20]. Then, neurons in the RVM project along the dorsolateral funiculus (DLF) to the dorsal horn[21].

The RVM mediates a bidirectional control of nociception by its resident ON- and OFF-cells. OFF-cells are thought to exert descending inhibition of nociception, while ON-cells seem to facilitate nociceptive mechanisms at the spinal dorsal horn[12, 19, 21]. These cells are not serotonergic but they can modulate serotonergic neurons of the NRM and the nucleus gigantocellularis. Once serotonin is released in the spinal dorsal horn, it plays inhibitory or facilitatory actions depending on the receptor subtype activated[22, 23]. Another important contributor of the modulatory control is the noradrenergic system, which is described more thoroughly in the next section.

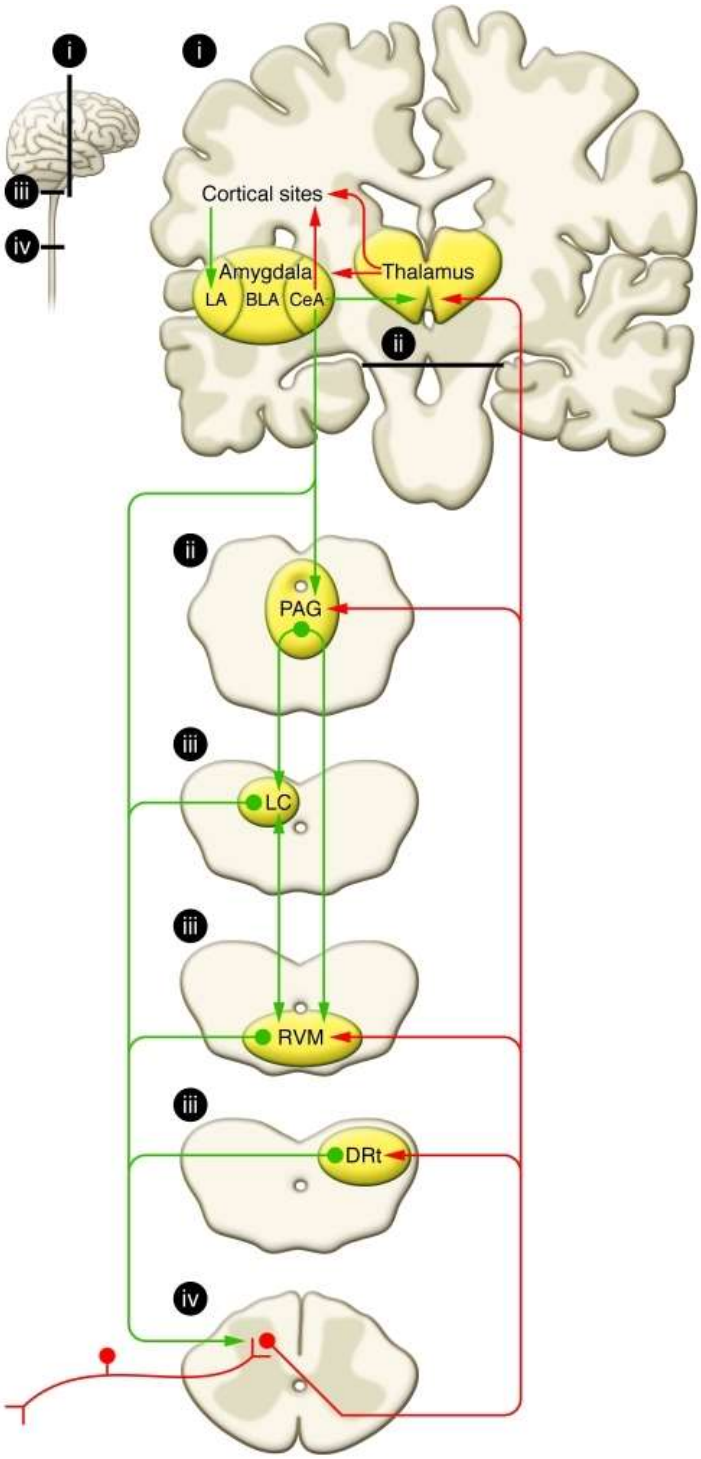


Figure 2- Schematic representation of pain modularity circuitry. Nociceptive inputs enter the spinal dorsal horn through primary afferent fibers that synapse onto transmission neurons. From dorsal horn, ascending projections, labeling in red, target the thalamus, and collateral projections also target mesencephalic nuclei, including the DRt, the RVM, and the midbrain PAG. Descending pain modulation is mediated through projections, labeling in green, to the PAG, which also receives inputs from other sites, including the hypothalamus (data not shown), and communicates with the RVM as well as other medullary nuclei that send descending projections to the spinal dorsal horn. The noradrenergic Locus Coeruleus receives inputs from the PAG, communicates with the RVM, and sends descending noradrenergic inhibitory projections to the spinal cord. Areas labeled "i–iv" in the small diagram correspond with labeled details of the larger diagram. BA – Basolateral amygdala; CeA – Central nucleus of amygdala; LA – Lateral nucleus of amygdala; (Adapted from Ossipov et al[12]).

1.3.2. The noradrenergic System

The noradrenergic system comprises a vital circuit based on the action of noradrenaline (NA) on different and strategically located adrenergic receptors. NA is involved in the autonomic regulation of various organs[24]. In the central nervous system (CNS), noradrenergic neurons are organized in seven clusters, classified from A_1 to A_7, which are located in the brainstem[25-27].

1.3.2.1. Locus Coeruleus

The *Locus Coeruleus* (LC or A_6), the main structure coordinating the central noradrenergic system, is located in the upper part of the floor of the fourth ventricle. Rather than being a single brain nucleus, the LC represents a nuclear complex that includes areas located bilaterally within the lateral pontine central grey, as well as scattered catecholamine neurons close to the *brachium conjunctivum* and within the central tegmental regions of the pontine gray matter[28, 29]. The main neurotransmitter synthetized in the LC nucleus is NA, but other substances are also produced in this structure.

Noradrenaline is a neurotransmitter biosynthesized by sequential enzymatic reactions starting with the amino acid tyrosine[24, 26]. This cascade starts with the conversion of tyrosine into dihydroxyphenylalanine (L-DOPA) through the rate limiting enzyme action of tyrosine hydroxylase (TH; Fig. 3)[24]. Then, L-DOPA is converted into dopamine through the action of the aromatic L-amino acid decarboxylase[26] and, only in noradrenergic neurons, dopamine is posteriorly converted into NA by dopamine-β-hydroxylase (DBH; Fig. 3). After this process, NA is stored into synaptic vesicles and in response to specific electrical input, these vesicles attach to the neuronal membrane through the vesicular monoamine transporter, releasing their content into the synaptic cleft[26, 30]. The NA present in the synaptic cleft can bind to post-synaptic adrenergic receptors and activate intracellular signaling cascades and, after transducing their specific signals, NA molecules can undergo reuptake by presynaptic NA transporters or they can be degraded by enzymes in the synaptic cleft and at the nerve terminal[26] (Fig. 3).

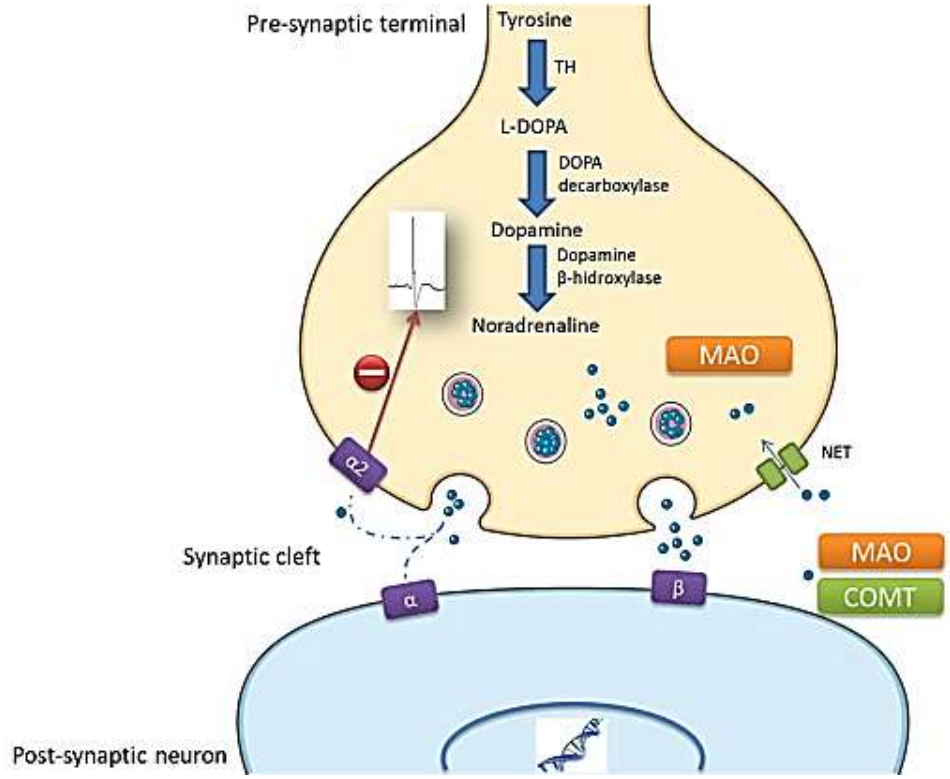


Figure 3- Schematic representation of a noradrenergic synapse. NA is synthesized from the amino acid tyrosine through a cascade of events. After its synthesis, NA is stored in vesicles at the axon terminal and following specific electrical input, these vesicles attach to the membrane and release the NA content into the synaptic cleft. In the extracellular space, NA exerts its function by binding to α and β adrenergic receptors in the post-synaptic membrane. When the flow of NA into the synaptic cleft is intense, α2-adrenoceptors in the pre-synaptic membrane (auto-receptors) are also activated and they inhibit NA release. At the same time, the extracellular NA content that does not bind to any receptor or that has already exerted its function may be recycled upon reuptake at the pre-synaptic membrane, or it may be degraded: L-DOPA – L-dihydroxyphenylalanine;COMT – Catecol O-Metiltransferase; MAO – monoamine oxidase; TH – tyrosine hydroxylase (adapted from M. Llorca-Torralba et al)[26].

Noradrenaline release exerts neuromodulatory effects on synaptic transmission by modifying the membrane potential, synaptic plasticity and the excitability of neurons via G-protein coupled receptors, α_1, α_2 and β (β_1, β_2 and β_3) adrenoreceptors (ARs)[29, 31]. The adrenergic receptors have a different distribution in the different targets of LC projections. The α_1 and β receptors are present primarily at postsynaptic sites, whereas α_2 receptors exist both pre- and postsynaptically[32]. In general, the action of these receptors is mediated by guanine nucleotide-binding regulatory proteins (G proteins) [26]. Although both categories have been implicated in pain modulation, the α-adrenoreceptors have a key role by mediating the pain regulatory effects of noradrenaline[26].

1.3.2.2. Descending noradrenergic modulation

The LC (or A_6), the A_5 and A_7 noradrenergic cell groups, are the main sources of noradrenergic projections to the spinal cord[25, 33, 34]. These cells groups are connected with other pain control centers and all of them receive projections from PAG[34]. LC also receives projections from the central nucleus of the amygdala (CeA), preoptic area, paraventricular nucleus of hypothalamus and lateral hypothalamus[35]. These noradrenergic projections form an important component of descending pain modulation. Electrical or chemical stimulation of noradrenergic nuclei, PAG and RVM produces spinal antinociceptive effects by releasing NA into the spinal cerebrospinal fluid.

At the spinal cord level, NA released from descending pathways may activate several pain inhibitory mechanisms. Direct catecholaminergic innervation of cell bodies of the spinothalamic tracts' neurons provide a structural basis for post-synaptic noradrenergic inhibition of spinal pain-relay neurons[25]. Noradrenaline also inhibits transmission of nociceptive signals in the spinal cord due to action on presynaptic α_2-AR[36].

Studies with a knockout for the *Dbh* gene that led to the absence of noradrenaline shows it has a minor effect on pain sensitivity[37], supporting the concept that noradrenergic systems have little influence on baseline pain sensitivity. However, during persistent pain it was shown that noradrenergic systems have a more important role[38, 39]. Recent studies suggest that in conditions of nerve injury, the descending noradrenergic system is augmented in an effort to compensate for enhanced nociceptive inputs. Injury is associated with increased synthesis and release of NA along with an enhanced efficacy of spinal α_2-AR[40].

The noradrenergic system is of high importance in the neuronal interactions responsible for pain modulation and is also involved in the regulation of emotional/affective behaviors. In fact, our group has established that noradrenergic activation by chronic pain increases the descending pain facilitation from the DRt[41-43].

2. Affective component of Pain

Pain comprises sensory, cognitive and, most importantly, affective components. The affective component includes feelings of annoyance, sadness, anxiety and depression in response to a noxious stimulus[44]. In chronic pain patients, mood disorders such as depression and anxiety are frequently observed, with prevalence rates around 30% in neuropathic pain patients[45, 46]. Although this comorbidity is clinically established, the underlying mechanisms still remain unclarified.

Clinical imaging studies of acute experimental pain in human subjects provided important information regarding the areas most commonly activated by noxious stimuli, including the primary somatosensory (S1) and secondary somatosensory cortex (S2), anterior cingulate cortex (ACC), insular cortex (IC), prefrontal cortex (PFC), insula, thalamus, amygdala and the mesolimbic reward circuit, which includes the ventral tegmental area (VTA) and nucleus accumbens (NAc). S1 and S2 activations contribute to the sensory-discriminative dimension of pain. The ACC, PFC, IC, NAc, and amygdala, meanwhile, have been implicated in the affective component of pain (Figure 4)[47].This interconnected group of brain regions also forms the basis for understanding the pathophysiology of depression. The NAc receives afferent nociception information through connections with thalamus, parabrachial area, amygdala and ACC. Corticostriatal connections from prefrontal, orbitofrontal and anterior cingulate cortices contribute to an affective, emotional and cognitive control of pain perception and are involved in motivational decision-making. In the NAc, glutamatergic outputs from the amygdala converge on dopaminergic terminals from the VTA and influence motivated behavior in response to stress and anxiety. The dopamine mesolimbic pathway, from the VTA to NAc, also attracted attention in depression research. It is a critical component of 'brain reward circuits' which also responds to aversive and nociceptive stimuli[48]. Additionally, a descending pathway from the NAc that can modulate spinal nociceptive signals, possibly via the RVM, has been suggested[49]. The most commonly studied pain modulatory pathways involve projections from the midbrain PAG to brainstem nuclei, including the rostroventral medulla (RVM) and the locus coeruleus, to the dorsal horn of the spinal cord[12, 50]. These pathways involve endogenous opioids, noradrenaline and serotonin, and have both inhibitory and excitatory actions on spinal cord afferent projection neurons[51]. Interesingly the monoamine serotonergic and noradrenergic systems have been associated with depression and its treatment. Alba-Delgado *et al.* explored the role of noradrenergic nucleus locus coeruleus to test the hypothesis that altered LC function

might promote depressive and anxiogenic behavior during chronic pain. An increased expression of TH and of NA transporter as well as an α_2-AR hypersensitivity has been observed in a neuropathic animal model, which affect LC firing and NA release in the prefrontal cortex, and is coincident with neuropathic pain-induced anxiodepressive behaviors[52]. It has also been recently postulated that long-standing chronic pain may promote higher predisposition for anxiodepressive states[53], not only associated with neuropathy but also with an inflammatory condition. Indeed, Borges et al. demonstrated in monoarthritic rats, that anxiety is associated with this inflammatory condition and is developed within a four-week period after the pain model induction. This finding was accompanied by exacerbated evoked LC responses to noxious stimulation of the inflamed and the healthy paw and increased phosphorylation (activation) of extracellular-signal regulated kinases 1/2 (ERK1/2) in the LC[54-56].

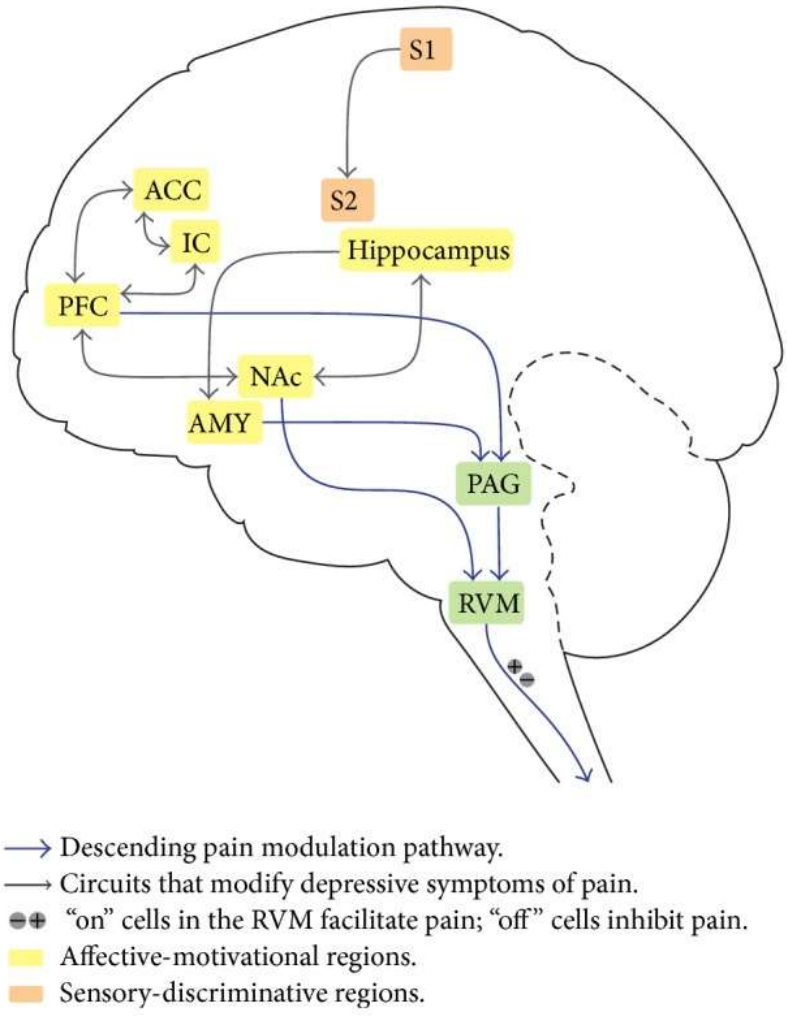


Figure 4- Brain regions and circuits implicated in the comorbidity between pain and depression. ACC: anterior cingulate cortex; AMY: amygdala; IC: insular cortex; NAc: nucleus accumbens; PAG: periaqueductal gray; PFC: prefrontal cortex; RVM: rostral ventromedial medulla; S1: primary somatosensory cortex; S2: secondary somatosensory cortex. (adapted from Doan, L., Manders, T., & Wang, J. (2015)[47].

3. Neuropathic Pain

The prevalence of chronic pain in Europe is about 25-30%[57] and is estimated that about a fifth of the persons who report chronic pain has neuropathic pain[58, 59]. Neuropathic pain is defined by IASP as “pain emerging as a direct consequence of a lesion or a disease of the somatosensory systems”[60]. This system is involved in the perception of touch, pressure, pain, temperature, position, movement and vibration. The somatosensory nerves are present in the skin, muscles, joints and fascia and include thermoreceptors, chemoreceptors, pruriceptors and nociceptors that send signals to the spinal cord and eventually to the brain where they will be further processed[61].

According to the location of the nerve lesion, neuropathic pain can be divided into two types, central and peripheral neuropathic pain.

3.1. Central and peripheral neuropathic pain

Central neuropathic is caused by a lesion or disease of the spinal cord and/or brain tissue[61, 62], including vascular (ischemic or hemorrhagic), infections (abscess, encephalitis, myelitis), demyelinating (multiple sclerosis), traumatic (brain or spinal cord), or neoplastic disorders. It can also result from syrinx formation in the spinal cord or brainstem[62]. Cerebrovascular diseases affecting somatosensory pathways (post-stroke pain) and neurodegenerative diseases are brain disorders that most commonly cause central neuropathic pain[63].

For some time, dysfunction of spino-thalamic-cortical pathways, clinically evident as impaired pain (pinprick) and temperature sensation, were considered critical in the development of central neuropathic pain[64-66]. However, the fact that not all patients with spinothalamic tract sensory loss suffer central pain suggests a required cofactor to drive its development. Increasing evidences suggest that this cofactor may be the denervation hypersensitivity of surviving spinothalamic tract axons.

By contrast, peripheral neuropathic pain is a condition that develops as a result of damage to the peripheral nervous system. Peripheral changes occur in primary afferent neurons (nociceptive small unmyelinated C-fibers and non-nociceptive myelinated A-fibers) after partial nerve lesion, leading to peripheral sensitization[67]. Painful generalized peripheral neuropathies, usually with symmetrical distribution, include those associated with *diabetes mellitus*, pre-diabetes, and other metabolic dysfunctions, infectious diseases, chemotherapy, immune and inflammatory disorders, inherited neuropathies and channelopathies[26].

Some axons are damaged and degenerate (upper two axons), whereas others (lower two axons) are still intact and connected with the peripheral end organ. The lesion triggers the expression of sodium channels on damaged C-fibers. Furthermore, products such as nerve growth factor that are associated with Wallerian degeneration are released in the vicinity of spared fibers, triggering channel and receptor expression (sodium channels, TRPV1 receptors, adrenoceptors) on uninjured fibers[68].

Indeed, some lesions or diseases of the somatosensory nervous system can lead to altered and disordered transmission of sensory signals into the spinal cord and the brain. Peripheral and central changes induced by nerve injury or peripheral neuropathy are summarized in figure 5.

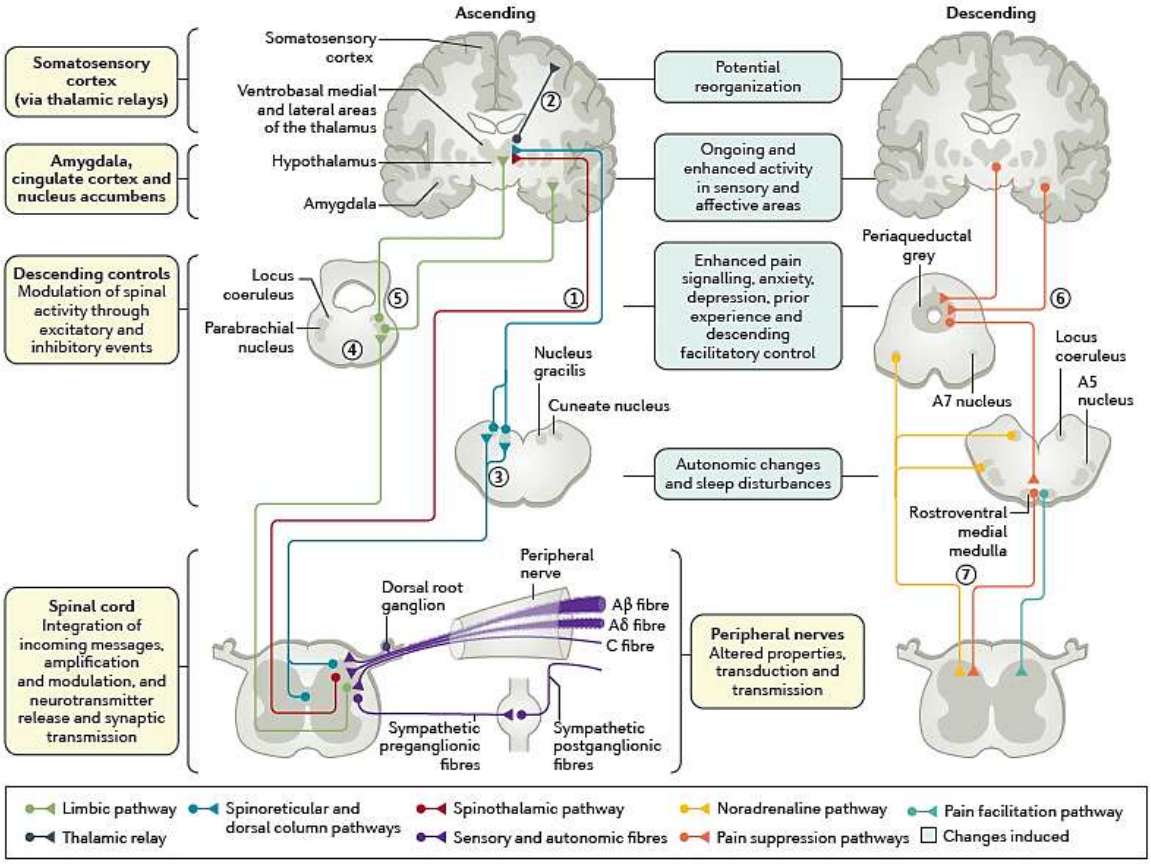


Figure 5-The peripheral and central changes induced by nerve injury or peripheral neuropathy. Damage to all sensory peripheral fibers alters transduction and transmission due to altered ion channel function. These alterations affect spinal cord activity, leading to an excess of excitation coupled with a loss of inhibition. In the ascending afferent pathways, the sensory components of pain are via the spinothalamic pathway to the ventrobasal medial and lateral areas (1), which then project to the somatosensory cortex allowing for the location and intensity of pain to be perceived (2). The spinal cord also has spinoreticular projections and the dorsal column pathway to the cuneate nucleus and nucleus gracilis (3). Other limbic projections relay in the parabrachial nucleus (4) before contacting the hypothalamus and amygdala, where central autonomic function, fear and anxiety are altered (5). Descending efferent pathways from the amygdala and hypothalamus (6) drive the periaqueductal grey, the locus coeruleus, A5 and A7 nuclei and the rostroventral medial medulla. These brainstem areas then project to the spinal cord through descending noradrenaline (inhibition via α_2 adrenoceptors), and, in neuropathy, there is a loss of this control and increased serotonin descending excitation via 5-HT_3 receptors (7). (adapted from Colloca L, Ludman T, Bouhassira D, et al.[17]).

3.2. Neuropathic pain treatment

Several recent studies have shown that NP can adversely affect patients' overall health-related quality of life, including physical and emotional functioning, as well as the mobility and ability to work, which entails substantial societal costs[69]. Treatment of neuropathic pain is challenging as only 50% of the treated patients experience satisfactory pain relief with drug side effects that are considerable. Patients with neuropathic pain continue to experience pain with moderate severity, despite taking prescribed medications[70]. The interest group NeuPSIG (Neuropathic Pain Special Interest Group) from IASP recommends as first line treatment, antidepressants, antiepileptics and topical lidocaine. Except in certain clinical circumstances, the use of opioids and tramadol is recommended as second-line treatment, principally for patients who have not responded to the first-line medications[70].

Secondary amines of tricyclic antidepressants (TCAs), including nortriptyline and desipramine, and antidepressants with both noradrenaline and serotonin reuptake inhibition (duloxetine, venlafaxine, milnacipran) act by increasing the synaptic serotonin and noradrenaline levels, potentiating the effect of the descending analgesic system[70-72]. Antiepileptic drugs, including carbamazepine and calcium channel α_2-δ ligands, such as gabapentin and pregabalin, function in several ways by modulating voltage-gated sodium and calcium channels, by enhancing the inhibitory effects of GABA and by inhibiting excitatory glutaminergic transmission[71]. Topical lidocaine is effective for areas of constant burning pain, especially if they are allodynic.

Tramadol is used as second-line treatment since it is an agonist of the opioid μ-receptor, but it also inhibits the reuptake of serotonin and noradrenaline. Although tramadol has demonstrated efficacy in several neuropathic conditions, it may be less efficacious than strong μ-agonists such as morphine and oxycodone[73].

4. Experimental Neuropathic Pain

The evaluation of neuropathic pain in humans is complex if we consider that most of the stimuli required to induce this condition produce irreversible damage. Furthermore, it is almost impossible to recruit a large number of humans for such type of clinical research. Therefore, to understand the mechanisms involved in neuropathic pain and to evaluate the analgesic potential of novel pharmacotherapies for the treatment of neuropathic pain, validated and reproducible animal models of neuropathic pain are necessary[74].

4.1. Animal models of neuropathic pain

Different models of neuropathic pain have been developed, mostly, in rodents (Table1). Some models aim to replicate peripheral NP mechanisms and others study central mechanisms[74]. These models are based on most of the known etiologies in humans, aiming to reproduce peripheral nerve injuries, central injuries, trigeminal neuralgia, diabetic neuropathies, chemo-induced neuropathies, postherpetic neuralgia, and so forth[74, 75]. Neuropathic pain models fit into three broad classes according to how pain is induced: i) traumatic nerve injury, ii) systemic neurotoxicity, or iii) other initiating injuries.

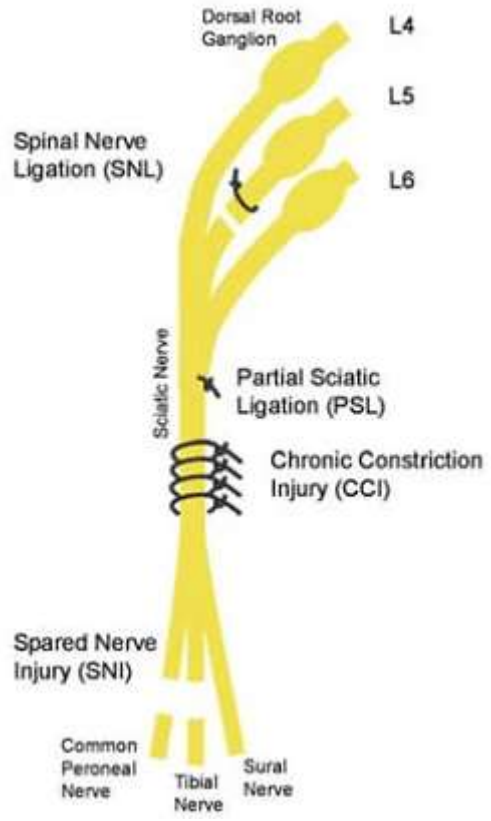


Figure 6- Animal models of Neuropathic Pain (adapted from Campbell and Meyer, 2006)

Traumatic nerve injuries typically constrict, ligate, crush or transect a nerve, producing a mixture of loss of sensation and gain of pain function. Four different nerve injury models are used[76] (Figure 6). In the spinal nerve ligation (SNL) model, one or more spinal nerves going to the foot are ligated and cut[77]. In the partial sciatic ligation (PSL) model, a portion of the sciatic nerve is tightly ligated[78]. The chronic constriction injury (CCI) model involves placement of four loose chromic-gut ligatures on the sciatic nerve which triggers an immune response to the sutures leading to nerve swelling and nerve constriction. The fourth nerve injury model is the spared nerve injury (SNI) and in this model, the common peroneal and tibial nerves are cut, while the sural nerve is spared[79]. In each model, only a portion of the afferents going to foot are lesioned. All types of injuries induced in these models lead to hyperalgesia, manifested by enhanced responses to mechanical, heat, and/or cooling stimuli. Concomitantly, in these neuropathic pain models, have been shown to induce depression-like behaviors.

Table 1-List of different animal models of neuropathic pain (adapted from Jaggi, A.S., et al.2011)[74]**.**

Model name	Type of injury	Animal
Axotomy	Complete sciatic section	Rat
Chronic sciatic constriction	4 ligatures around the nerve	Rats and mice
Partial sciatic ligature	Ligature of 1/3 to ½ the nerve	Rats and mice
Spinal roots ligation	1. Ligation of L5/L6	Rats
	2. Ligation of L7	Monkeys
Nerve-sparing injury	Tibial and peroneal axotomy	Rats and mice
Tibial and sural nerve transection	Tibial and sural axotomy	Rats
Common peroneal ligation	Common peroneal ligation	Mice
Sciatic cryoneurolysis	Nerve freezing	Rats
Caudal trunk resection	Caudal trunk resection	Rats and mice
Sciatic inflammatory neuritis	Zymozan injection, TNF around the nerve	Rats and mice
Balloon-induced sciatic injury	Implant of polyethylene balloon around the nerve	Rats and mice
Laser-induced sciatic injury	Decreased blood flow to the nerve mediated by radiation	Rats
Spinal injury by contusion	A weight is dropped on exposed spinal cord	Rats and mice
Excitotoxic spinal cord injury	Spinal injection of aminoacids	Rats and mice
Spinal hemisection	Laminectomy of T11-T12	Rats
Drug-induced	Direct drug injury to peripheral nerves	1.Rats, mice, Guinea pigs
1. antineoplastic drugs (vincristine, cisplatin,		
oxaliplatin, paclitaxel)		
2. anti-HIV (2,3- dideoxycytidine, didanosine)		2. Rabbits, rats
Diabetes-induced neuropathy	Persistent changes in nerves induced by hyperglycemia	Rats and mice
1. induced by streptozocin		
2. genetic models		
Bone pain models	Inoculation of cancer cells in bones	Rats and mice
HIV-induced neuropathy	Inoculation of HIV gp120 protein in sciatic nerve	Rats
Postherpetic neuralgia	Injection of viral cells	Rats
Alcoholic neuropathy	Ethanol administration for long periods	Rats
Pyridoxine-induced neuropathy	Administration of high doses of pyridoxine for long periods	Dogs and rats
Trigeminal neuralgia	Trigeminal compression; chronic infraorbitary nerve constriction	Rats
Orofacial pain	Formalin of carrageenin injection in temporomandibular joint and jaw	Rats and mice
Acrylamide-induced	Administration of acrylamide for long periods	Rats

4.2. Spared nerve injury

In the SNI model, developed by Decosterd and Woolf[79], the tibial and the common peroneal nerves are sectioned, leaving the sural nerves intact[74] (Fig. 6). This procedure causes a chronic behavioral mechanical and thermal hypersensitivity of the lateral surface of the hindpaw (sural nerve skin area). The areas of the spared sural nerve, and in less extent the saphenous nerve, are both affected, but no changes are observed in the contralateral hindpaw. This change in sensitivity of the intact fibers adjacent to a transected nerve likely results from denervation of the skin normally innervated by the tibial and common peroneal nerves, and by consequent Schwann cells denervation. The difference observed between the saphenous and sural territories is due to the fact that the saphenous nerve is a part of the femoral nerve plexus and enters the spinal cord via a set of dorsal root ganglia different from those of the sciatic nerve[79].

5. Genetic manipulation of the nociceptive system

The limited therapeutic efficacy and major undesirable side effects frequently associated with current pharmacological treatments of pain explain a strong need of novel therapeutic approaches[80-82]. Current pharmacological and interventional modalities available for the treatment of chronic neuropathic pain have variable efficacy and some current therapies include intolerable adverse effects[83]. The development of gene therapy has opened the possibility of using nonviral or viral vectors to transduce genes that encode antinociceptive substances to treat chronic pain and study the nociceptive system[81, 84]. An ideal gene therapy should be efficient in delivering exogenous genes to target cells that mediate high level and long-term gene expression, and an ideal gene therapy vector should not be able to replicate its own DNA, and should be conductive to long-term gene expression, nonpathogenic and nontoxic[85]. Viral vectors are generally created by deleting nonessential genes from the virus while remaining the structural motifs necessary to transfer its genome into the host[81, 86]. Herpes simplex Virus (HSV), adeno-associated virus (AAV), adeno virus (AV), lentivirus (LV) and human foamy virus (HFV) can be used as viral vectors for gene therapy of chronic pain. These viral vectors are the most commonly used because of their immunogenicity, natural integration ability and other features. Herpes simplex virus has neurotropic features and naturally maintains lifelong residency in the nucleus of infected neurons, making it suitable for transduction in the nervous system[81, 87].Adeno-associated viral vectors are commonly used to deliver therapeutic genes to target tissues with lower immunogenicity[81, 88]. They have a gene carrying capacity of 4.5 kb and can transduce both dividing and nondividing cells[89]. These viral vectors cannot integrate into the host genome, so there is a low risk of insertional mutagenicity[90]. Lentiviral vectors have the advantage of long-term transgene expression, low immunogenicity, and the ability to accommodate larger transgenes[91]. LVs belong to a subclass of retroviruses that integrate into the host cell genome. Due to their natural integration ability, LVs have been extensively utilized for ex vivo gene transfer because of their strong tropism for neuronal stem and progenitor cells[92]. HFV was the first identified human retrovirus and is nonpathogenic and has several unique features related to gene transfer, making it a promising vector system for gene therapy[93].

Table 2 - Viral vector systems[1] (adapted from Howarth, J. L., Lee, Y. B., & Uney, J. B. (2010)) [94].

Vector	Entry	Immune response	Capacity (kb)	Location	Ease of production	Expression
Retrovirus (inc lentivirus)	Fusion	Minimal	8	Integrates	10^{8}**	Years
Adenovirus	Receptor	Brisk***	36	Episomal	10^{12}*****	Weeks/months
AAV	Receptor	None	4	Integrates	10^{8}***	Years
HSV	Fusion	Brisk****	30	Episomal	10^{10}****	Weeks

The use of viral vectors in neurobiological research has been growing due to their increasing flexibility and the ability to deliver viral particles to brain regions[85]. In the last decades, genetic manipulation has been helpful for a better understanding of the molecular mechanisms of the descending pain modulatory system[11, 95]. It may also become a versatile tool for chronic pain management, based in three parameters, the vector, the transgene and the promoter. The best combination of these parameters allows to design a gene therapy strategy to study chronic pain[11]. The vector is the carrier of the transcriptional cassette and its main function is to deliver its content to specific cell targets. Some of them have the ability to be transported retrogradely, which allows the vector to be uptaken at the nerve terminal and then migrate to the nucleus, often located in remote areas, surgically difficult to access[96].

Recent studies targeting the noradrenaline pathway by using gene therapy, specifically noradrenaline synthesis inhibition, allowed a novel approach to pain treatment. Martins et al[42] successfully demonstrated a significant decrease in noradrenaline associated with gene expression in the DRt and also a significant inhibition of mechanical and cold allodynia and mechanical hyperalgesia by using HSV-1 as a vector for delivery of the tyrosine hydroxylase transgene inserted in an antisense orientation into the DRt of the rat brain. This study opened new potential ways to target the noradrenergic modulation during neuropathic pain conditions[83].

The understanding of pain-signaling pathways and the specific nerve circuits involved is of major importance for possible therapeutic interventions. Several techniques, classically based on various anterograde and retrograde tracers, electrophysiological recording, viruses and more recently, numerous fluorescent tracers, plant lectins, toxins and fusion proteins, were used for mapping, starting at the periphery and terminating in cortical areas. Although development of approaches allowing the passage of tracer systems across the synapses was an important evolution, the real break-through in this field was the set up of transgenic systems based on inducible tracer expressions. The

[1]Asterisks indicate the relative ease of production or the relative level of immune response, i.e. a greater ease of production or greater immune response elicited is represented by a higher score

most current conditional gene-targeting systems are based on the use of the site-specific recombinase Cre (*c*yclization *re*combination) which catalyzes recombination between two 34-bp DNA recognition sites named loxP (*lo*cus of crossing [*x*-ing]-over of bacteriophage *P*1). The basic strategy for Cre-lox-directed gene Knockout experiments is to flank, or "flox", an essential exon of the gene of interest with two loxP sites (by homologous recombination in embryonic stem cells), and then to "deliver" Cre that excises the intervening DNA including the exon from the chromosome, thus generating a null allele in all cells where Cre is active. The bacterial Cre recombinase activity may be provided either by crossing mice carrying the floxed target gene with transgenic Cre-expressing mice or by using viral vectors expressing the Cre transgene[97].

II. Aims

The LC is the principal noradrenergic nucleus in the CNS and is the main source of noradrenergic innervation to the spinal dorsal horn. The LC also projects almost to the entire neuraxis and plays a pivotal role in diverse behaviors such as learning and memory, motivation and emotions with LC-derived noradrenaline being linked to aversive states like stress, anxiety and depression. However, the mechanisms and neuronal pathways linking neuropathic pain and depression/anxiety are still unknown and solid and reliable information is still quite sparse. This project aims at providing insight on the effects of the noradrenergic system on nociception and chronic pain-induced anxiety and depression during neuropathic pain conditions. For that purpose, it is imperative to optimize all procedures including the animal model. By using temporal and spatial conditional knockout (CKO) mice for the noradrenaline biosynthetic enzyme Dbh at the LC, and the SNI model of neuropathic pain, the main objectives of this thesis were:

1. To establish a protocol for *Dbh* gene ablation at the LC of transgenic mice for the successful generation of a temporal and spatial conditional KO animal model for NA production at the LC, to be used in this project;
2. To optimize the lentiviral stereotaxic injection conditions to allow complete *Dbh* ablation at the LC (viral particles concentration, injection volume, stereotaxic coordinates and latency time for maximum virus expression).
3. To study the effects of *Dbh* KO at the LC, and therefore of NA impaired synthesis, on the induction and development of neuropathic pain;
4. To study the effects of *Dbh* KO at the LC, and therefore of NA impaired synthesis, on the depressive and anxiety-like behaviors during neuropathic pain;

III. Materials and Methods

1. Generation of mutant mice

Mutant mice allowing the conditional knockout of the *Dbh* gene by the Cre-Lox recombination system were developed by members of our Laboratory of Support to Research in Molecular Medicine (LAIMM) following the steps 1.1, 1.2 and 1.3, as follows:

1.1. Generation of chimaeric mice and germ line transmission of the *Dbh* ^{tm1a} allele

Ready-targeted Dbh $^{tm1a(EUCOMM)Wtsi}$ ES cells (clone EPD0800_2_E05, *Dbh* knockout-first with conditional potential allele, European Conditional Mouse Mutagenesis Program-EUCOMM Consortium) were microinjected into C57BL/6J 8-cell uncompacted morulae using a Leica AM6000 Advanced Micromanipulation system.

Injected embryos were implanted into the uteri of pseudopregnant CD-1 females and the born chimaeric animals were readily identified by the coat colour (*Agouti* in white CD-1 background). Chimaeric males were mated with C57BL/6J females to obtain germ line transmission of the *tm1a* allele. Heterozygous mutant progeny was easily discriminated by the *Agouti* coat color and following molecular genotyping by PCR of genomic DNA isolated from ear punch or tail biopsies at weaning.

1.2. Generation of the Dbh $^{flox/flox}$ allele

The knockout-first *tm1a* allele was converted into the floxed *Dbh* allele (*Dbh* $^{flox/flox}$) following recombination *in vivo* by crossing the heterozygous mutant with a constitutive *FLP* deleter strain (B6.129S4-Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$/RainJ, The Jackson Laboratory). Successful recombination of the FRT sites by the *FLP* recombinase was confirmed by PCR in the double heterozygous progeny. Experimental Dbh $^{flox/flox}$ homozygous mice, as well as control wild type littermates, were obtained from heterozygote intercrosses.

1.3. PCR genotyping

Genomic DNA isolated from ear punch or tail biopsies was PCR amplified using two sets of primers as follows: a) detection of the *tma1* (KO-first allele) - Pair 1, Fw GGGGAAACTCAAACACTGCT, Rv ACCACCTCATCAGAAGCAGG, 60 ºC annealing, 35 cycles; b) detection of *Dbh* floxed allele - pair 2, Fw GGGGAAACTCAAACACTGCT, Rv GGAGGCAGGGGAAAGGTATT, 60 ºC annealing, 35 cycles. Primer pair 1 produces an amplicon of 313 bp encompassing endogenous sequence and part of the *tma1* targeting cassette; the WT does not amplify. Pair 2 anneals to genomic sequence only amplifying a fragment of 118 bp within the *Dbh* locus of wild type animals and a fragment of 298 bp encompassing a LoxP site and remainings of the targeting cassette flanked by the endogenous sequence in *floxed* animals; the *Tm1a* allele (KO-first) does not amplify.

1.4. Generation of spatial and temporal conditional KO mice for Dbh (Dbh-CKO)

Experiments were carried out in the Dbh $^{flox+/-}$, Dbh $^{flox-/-}$ or the Dbh $^{flox/flox}$ homozygous C57BL/6 mice (now named as Dbh $^{flox+/+}$) generated as described above. The Dbh $^{flox+/+-}$ mice were used for the spatial and temporal generation of conditional KO mice for Dbh (Dbh-CKO). The conditional knockdown of the *Dbh* gene was induced at a specific time point of the experiments and was circumscribed specifically to the LC area by using the Cre-LoxP recombinant system, and by injecting a lentiviral vector possessing the Cre recombinase gene (LV-Cre: hSyn-eGFP-Cre) specifically into the LC region.

Animals were hosted in *Biotério Geral do Centro de Investigação Médica da Faculdade de Medicina da Universidade do Porto* under controlled conditions of temperature and humidity, 23 ± 2ºC and 50 ± 10%, respectively, with food and water freely available and darkness/light periods of 12 hours.

The experimental procedures were performed in accordance with the ethical guidelines for the study of experimental pain in conscious animals[98], and the European Council Directive 2010/63/EU, and were approved by the Ethical Committee for Animal Experimentation at our institution (ORBEA) and by *Direção Geral de Alimentação e Veterinária* (DGAV), Portugal. The mice physical condition was monitored throughout the experiment, with attention to the presence of stress signs, illness or poor physical condition, such as loss or gain of excessive weight, dehydration, aggressive social behavior, low mobility, bleeding and poor wound healing, infection of the sutures and opening of the stitches in the post-surgery period.

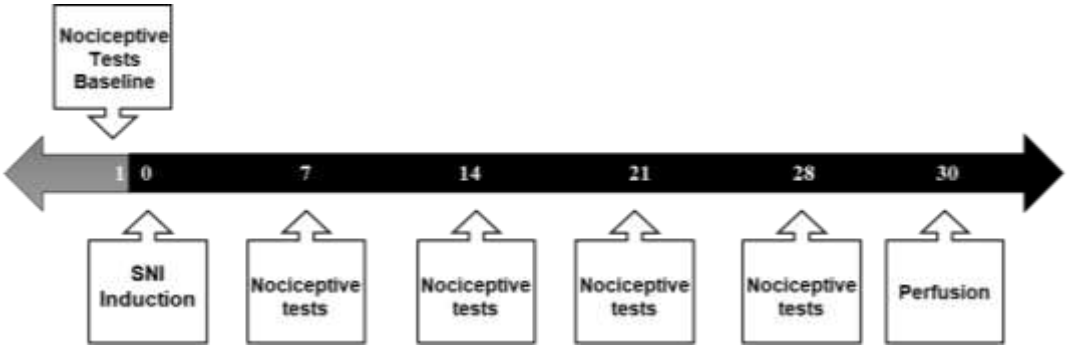
2. Experimental design

Experimental procedures comprised three different groups of mice weighting between 25 to 30g. Groups 1 and 2 were used as controls and Group 3 served as the experimental group. All mice were submitted to surgical and behavioral assessment procedures as described in detail below. At the end of the experimental period, all the animals were sacrificed for histological and molecular analyses.

Experimental group 1 consisted of Dbh $^{flox+/-}$ or Dbh $^{flox-/-}$ mice that underwent either sham (N= 7) or spared nerve injury (SNI) surgeries (N= 5), and were then subjected to anxiety and depression-like behavior tests at six weeks after the surgeries. This was followed by nociceptive behavior tests, which were performed a week later, to ensure that all the animals had developed the typical hypersensitivity symptoms associated with a neuropathic pain condition (Scheme 1).

Scheme 1 - Experimental Group 1 used for preliminary evaluation of anxiety- and depression-like behaviors in neuropathic pain animals, and as a control of the experimental conditions in the for anxiety and depressive-like behavioral tests.

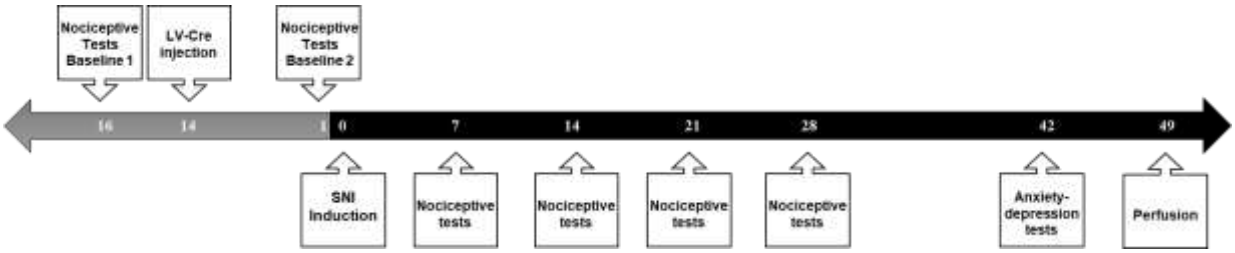
In Experimental group 2 (N=5), mice with the Dbh $^{flox+/+}$ genotype were subjected to the SNI surgery to induce a neuropathic pain condition. Nociceptive behavior tests were performed before the SNI surgery (baseline) and then once a week for 28 days (Scheme 2).



Scheme 2 - Experimental Group 2 was a control for neuropathic nociceptive behavior and the evolution of this condition throughout time.

Experimental group 3 (N=8) was used for the evaluation of the efficacy of conditional Dbh ablation at the LC of the mice, and of the effects on the nociceptive and anxiety-

and depression-like behaviors in those mice upon induction of a neuropathic pain condition. For this, animals with the Dbh $^{flox+/+}$ genotype underwent first a stereotaxic injection for delivery into the LC of a lentivirus containing the Cre recombinase gene (LV-Cre: hSyn-eGFP-Cre) in order to induce the *Dbh* gene ablation. Fourteen days later, they were submitted to the SNI surgery to induce the neuropathic pain model. Nociceptive baselines were obtained two days before stereotaxic injection (Baseline 1) and one day before spared nerve injury induction (Baseline 2). Then, the behavioral tests were conducted once a week for 4 weeks. The development of anxiety- and depression-like behaviors was also studied. After the last nociceptive behavioral test, mice were allowed to repose for two weeks. This period was planned to reduce stress and other environmental factors that may interfere with behavioral testing. Then at the 6 weeks' time point after SNI induction (42 days), mice were submitted to anxiety and depressive-like behavior testing (Scheme 3).



Scheme 3 - Experimental Group 3 was the main experimental group with Dbh gene ablation.

3. Lentiviral vectors

To knockout the DBH floxed gene we used a lentiviral vector purchased from the Gene Vector and Virus Core of the Stanford University (CA, USA). The lentiviral vector named LV-Cre carries an expression cassette that contains the human synapsin promoter (hSYN-p), the Cre recombinase cDNA coupled to the enhanced green fluorescent protein (EGFP) reporter gene and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE; Scheme 4). The WPRE was used to improve transgene expression. The lentiviral particles were produced by transfection of human embryonic kidney 293T cells with the LV-Cre vector, a packaging plasmid (pCMVΔR8.92), a plasmid encoding the rev protein (pRSV-Rev) and a plasmid encoding the vesicular stomatitis virus G glycoprotein (pMD.G).

Scheme 4 - Schematic diagrams of the LV-Cre vector. The lentiviral vector contains the human synapsin promoter (hSYN-p), the coding region of EFGP coupled to Cre recombinase in the same orientation) and the woodchuck hepatitis virus post-transcriptional regulator.

4. Surgical Procedures

4.1. Stereotaxic Surgeries

At the start of the surgical procedures, mice weighing 25 to 30g were deeply anesthetized with an intraperitoneal injection of a mixture containing ketamine hydrochloride (IMALGENE™ 1000, Merial, Chile; 60 mg/kg) and medetomidine (Medetor, Virbac; 10 mg/kg) and were placed on a rat stereotaxic frame with a mouse adapter (Stoelting Instruments, USA) for bilateral injection of lentiviral vectors (LV_Cre) into the LC by using a Hamilton syringe. Preliminary experiments aimed at optimizing the procedure conditions, namely the accurate stereotaxic coordinates, volume and concentration of injected LV-Cre virus and latency time for maximum lentivirus expression, were firstly performed as described below.

4.1.1. Optimization of the stereotaxic injections protocol

a) <u>Establishment of accurate LC coordinates for injections</u>

After identifying a brain region to be studied, coordinates for stereotaxic injections must be optimized. Firstly, it was necessary to determine initial coordinates (anteroposterior (AP); lateromedial (LM); dorsoventral (DV)) by locating the injection target in the brain atlas Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates (4th Edition). The chosen initial coordinates were: anteroposterior (AP) -5.3mm, lateromedial (LM) +/- 1.0, dorsoventral (DV) 3.6mm in relation to bregma.

Then a sky-blue dye was injected bilaterally at LC at the determined coordinates followed by animal sacrifice by decapitation under deep anesthesia in order to verify if these theoretical coordinates were on the desired target. The coronal sections surrounding the region of injection were collected and stained with formol-thionin for microscopic observation. For that tissue sections encompassing the LC were obtained in a cryostat at 30µm and mounted on poly-L-lysine coated glass slides, the slices were dried for 1h at 37ºC and immersed in a solution containing 4 volumes of acetone to 1 volume glacial acetic acid for 5 minutes. Washes were made with distilled water then the sections were stained for a period of 20 seconds in a formol-thionin solution (0,1g of thionin in 100mL

of a 10% formaldehyde solution). Finally, the material was washed again in distilled water, dried at 37ºC during one overnight or at least 1h, cleared in Xylene and mounted with Eukitt quick-hardening mounting resin (Jeulin, France) and cover-slipped. Several adjustments of the coordinates were performed using several animals until consistent and accurate LC targeting was achieved., After optimization of the coordinates, the mutant mice were injected with 0.4µL of LV13 with 1x106 IV/mL, per injection in two rostrocaudal areas of both the left and right LC with the following coordinates in relation to bregma (1st injection: anteroposterior (AP) -4.80mm; lateromedial (LM), +/-0.80mm; dorsoventral (DV), -3.90mm; 2nd injection: AP, -4.90mm; LM, +/- 0.80mm; DV, 3.70mm). The vectors were slowly injected (flow rate:0.1µL/2min) and, at the completion of the injections, the needle was left in place for 10 min before being slowly and carefully removed.

b) Determination of viral vector concentration

After coordinates standardization, we aimed at determining the lentiviral concentration (titer) allowing the best levels of lentiviral transduction. Far that, 0.4µL of LV-Cre was injected into the LC of the mice following the newly optimized coordinates at two concentrations ($1x10^5$ IV/mL and $1x10^6$ IV/mL). The lentiviral transduction was determined through analysis of the (1) EGFP expression in LC brain slices, and (2) the efficacy/level of *Dbh* gene ablation (immunoreaction against DBH).

c) Determination of the minimum time required for Cre-recombinase expression and *Dbh* ablation

The choice of the latency time after lentivirus injections to allow the expression of the Cre recombinase gene and complete *Dbh* gene ablation is another crucial factor for our experimental setting. For this, after LV-Cre stereotaxic injection, the mice were allowed to rest for 10 days, time considered necessary for efficient gene expression from lentiviral vectors[82]. To verify *Dbh* gene ablation, DBH protein expression at the LC was evaluated in the brain sections through immunohistochemistry essays.

4.2. Spared nerve injury

Fourteen days after LV-Cre injection, the SNI surgery was performed on the left hind paw of each mice, under isoflurane® anesthesia (2.5% for induction, 1.5% for maintenance), as previously described [79]. Briefly, the skin of the lateral surface of the thigh was incised and the muscle layers of the *biceps femoris* muscle were carefully opened, exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. The SNI procedure comprised an axotomy and ligation of the tibial and common peroneal nerves leaving the sural nerve intact. The common peroneal

and tibial nerves were tight-ligated and sectioned distally to the ligation, removing 1-2 mm of the distal nerve stump. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The muscle and skin were then sutured in two layers. In sham controls there was the exposure of the sciatic nerve and its branches without any lesion[79].

5. Behavioral Assessment

Testing procedures started with an acclimatization period of the animals to the researcher and to the testing room environment, followed by a habituation to the testing equipment. Behavioral tests were performed always in the same order for each animal (nociceptive tests: 1st Von Frey; 2nd Acetone. Anxiety and depression tests: 1st Open Field -OF; 2nd Elevated Plus Maze -EPM; 3rd Marble Burying -MB; 4th Forced Swimming Test -FST) at the following time points previously described in schemes 1-3. The anxiety- and depressive-like behavior tests were performed always during the weekend to avoid biases on the data resulting from perturbation/stress of the animals.

5.1. Nociceptive Tests

After SNI induction, the animals typically develop hypersensitivity to mechanical stimuli on the injury paw and cold allodynia[79]. To confirm the development of hypersensitivity, after a training period for habituation purposes, mice were placed on an elevated mesh platform, and the von Frey filaments were inserted through the mesh allowing the stimulation of the lateral plantar surface of the injured (left) hind paw[43]. Cold allodynia was determined by the application of a drop of acetone and the duration of the withdrawal response (paw withdrawal latency) was recorded.

5.1.1. Von Frey

The von Frey test (Figure 7 A and B) was performed by the application of calibrated monofilaments (Figure 7B), in a sequence of increasing forces (0.008, 0.02, 0.04, 0.07, 0.016, 0.40, 0.60, 1.0, 1.4, 2.0 and 4.0 g). Monofilaments were perpendicularly applied to the paw with sufficient force to cause filament bending. Ten stimuli were performed with 5 seconds intervals between the 10 consecutive filament applications and 30 seconds intervals between different von Frey filaments. Testing started with the lower filament (0.008g) and the number of positive hind paw withdrawal responses occurring in ten applications of each monofilament of the series was counted. The filaments were tested in ascending order of intensity to determine the relative frequency of paw withdrawal[99].

Figure 7- Illustrative image of the von Frey nociceptive test. A) Elevated mesh platform; B) Von Frey monofilaments.

5.1.2. Acetone Test

The acetone test evaluates cold allodynia. The response to the cold stimulus was evaluated by applying an acetone drop (10 µL) with a micropipette to the plantar skin of mice's left hind paw[100]. The observed effect was classified according to a scale ranging from 0 to 4 (0 = no response; 1 = no hind paw withdrawal, but sniffed the paw; 2 = Paw withdrawal for less than 5 seconds; 3 = Paw withdrawal from 5 to 30 seconds with sniffing, shaking and/or licking the paw; 4 = Paw withdrawal for more than 30 seconds with sniffing, shaking and/or licking the paw)[101, 102].

5.2. Anxiety and Depression Behavior Tests

In order to evaluate the development of anxiety and depression, behavioral tests were implemented at 2 weeks after the nociceptive tests. To evaluate anxiety-like behaviors the marble burying test and the elevated plus maze were used. The depressive-like behavior was evaluated through the modified version of the forced swimming test, and the open field test was used to analyze anxiety generated in exposure to new environments and, also, to exclude any locomotor impairment of the animals that could influence the results obtained in the other tests.

5.2.1. Open Field

The open field activity measurement is a method to assess anxiety-like and exploratory behaviors, in particular locomotive impairment (Figure 8 A). Briefly, mice were placed for 10 minutes in the center of a plastic (polyvinyl) square chamber (45cm wide x 45cm deep

x 40cm height). The open field arena was divided into a grid of three equally sized areas (center area, internal periphery and external periphery) that allows scoring the activity and distance travelled. During the test, the behavior of each mice inside the open field arena was recorded with a video camera and the videos were posteriorly analyzed using the SMART® *v3.0.03* (Panlab, Harvard apparatus) software. Rodents will typically spend a significantly greater amount of time exploring the periphery of the arena, usually in contact with the walls (thigmotaxis), than the unprotected centre area. Mice that spend significantly more time exploring the unprotected centre area demonstrate anxiolytic-like baseline behavior[103].

5.2.2. Elevated Plus Maze

The elevated plus maze test (Figure 8 B) is used for measuring the anxiety-related behavior and is based on the natural aversion of mice for open and elevated areas. The test consists of a platform elevated 30 cm from the floor, divided in open arms and closed arms which cross in the middle perpendicularly to each other, and an opened center area, as previously described[104]. Each mouse was placed individually at the center with its head directed towards an opened arm, with free access to the different arms. The behavior of each mice in the EPM was recorded during 5 minutes using a video camera and the videos were then analyzed using the SMART® *v3.0.03* (Panlab, Harvard apparatus) software. The time spent in the center, open and closed arms and the total distance travelled was quantified. A large amount of time spent in closed arms is associated with anxiety-related behaviors[104].

5.2.3. Marble Burying

The Marble burying test (Figure 8 C) allows evaluating the anxiety-related behavior. As previously described, mice were placed, for 30 minutes, into a transparent plastic cage with appropriate bedding (3-5cm height), containing 20 glass marbles arranged in four columns and five rows. During the 30 minutes of the test, mice were constantly exposed to a direct light of 100W at 50cm from the top of the cage. The reflection created by the light over the marbles causes an aversive stimulus for the mouse inside the cage leading the animal to bury the marbles on the bedding. After 30 minutes, the amount of marbles covered with bedding in at least 2/3 of their volume were considered as buried and were counted. A higher number of buried marbles is associated with increased anxiety-related behavior[105, 106].

5.2.4. Forced Swimming Test

The forced swimming test (Figure 8 D) is used to evaluate the depressive-like behavior. For this test, transparent cylindric tanks (30 cm height x 22.5 cm diameter) were filled with tap water until 15 cm from the bottom and its temperature was maintained at 23-25 ºC. Mice were gently and slowly placed in the water for 6 minutes and filmed to score the times spent in immobility (floating without struggling) and mobility (such as swimming and climbing) behaviors. At the end of the six minutes of testing, the recording was stopped and video analyses were performed using SMART® *v3.0.03* (Panlab, Harvard apparatus) software to quantify the time spent in immobility or mobility behaviors. Only the last 4 minutes of the test were analyzed, due to the fact that most mice were very active at the beginning of the test[107].

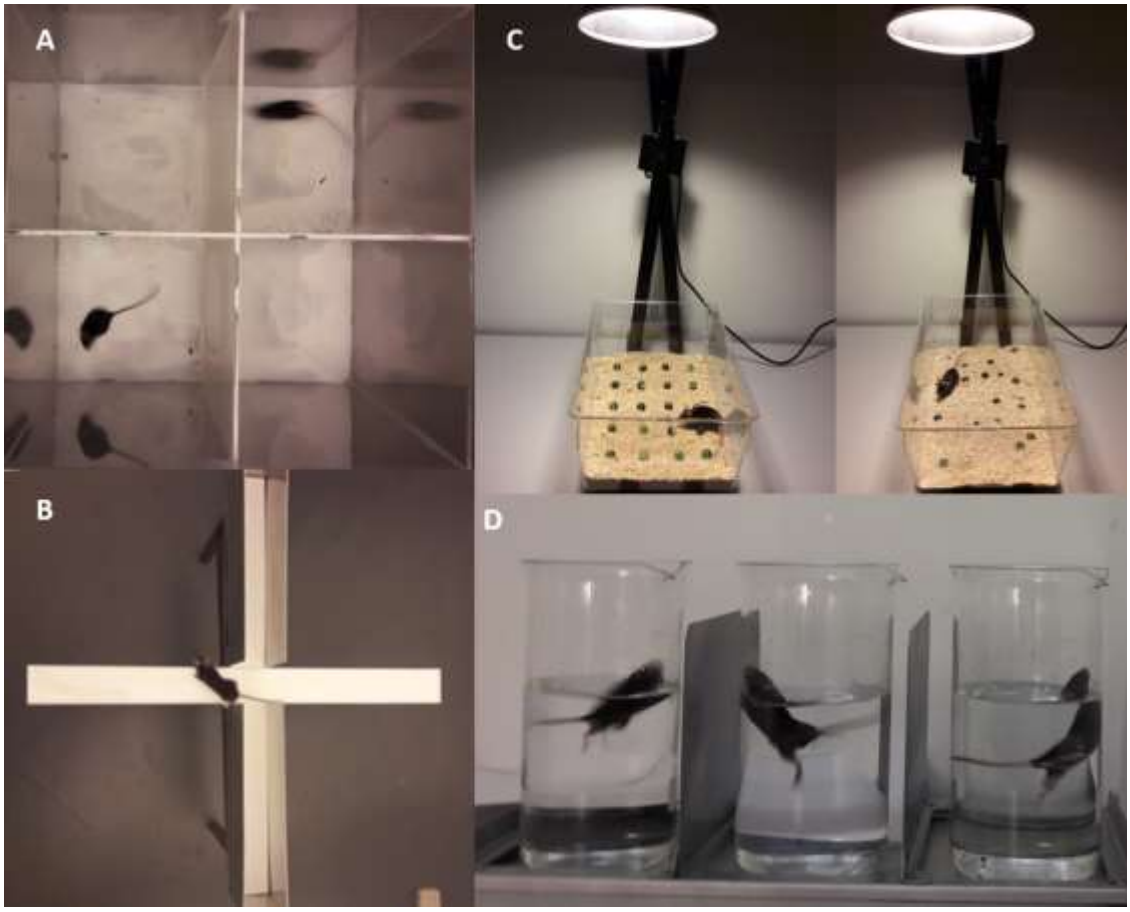


Figure 8 – Illustrative image of anxiety and depression Tests. (A) Open Field box; (B) Elevated Plus Maze; (C) Marble Burying before and after test; (D) Forced Swimming test.

6. Tissue preparation and Immunohistochemistry

After the behavioral tests, mice were deeply anaesthetized with sodium pentobarbital (50 mg/Kg intraperitoneal) and perfused through the left ventricle with 120 mL of calcium-free Tyrode's solution, followed by 120mL of a fixative solution containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.2.

The spinal cord and brainstems were removed, immersed in the same fixative for 4h, followed by 30% sucrose in 0.1 M phosphate-buffered saline (PBS) overnight, at 4ºC and then cryopreserved until they were processed. For DAB (diaminobenzidine) immunoreaction, the spinal cord and brainstem were sequentially sliced at 30 µm in a freezing microtome (Leica CM1325 microtome, Leica Biosystems® with a Huber Ministat 240 thermostat, Huber®) and preserved in a cryoprotectant solution at -20ºC. For immunofluorescence labelling the spinal cord and brainstems were sequentially sliced in 14 µm thick sections using a cryostat (Leica instruments GmbH, CM3050, Germany), and collected into poly-L-Lysine coated glass slides. After cutting, the slides were dried on a heating plate for 1 hour at 37ºC and thereafter were stored at -20ºC until use in immunohistochemical reactions.

6.1. Immunoreaction against DBH

Immunohistochemistry using diaminobenzidine (DAB) as chromogen

The slices containing the *Locus coeruleus* (LC) were washed firstly in PBS 0.1M. After inhibition of the endogenous peroxidase activity by incubation with a solution of 1% hydrogen peroxide in PBS and further washes in PBS and PBS-T (0.1 M PBS containing 0.3% Triton X-100), sections were incubated for 2 h in a blocking solution containing 0.1 M glycine and 10% normal goat serum (NGS) in PBS-T. Then, sections were incubated for 1 h at room temperature followed by 48 h at 4º C with a specific antibody against DBH (ImmunoStar, AB572229, anti-DBH raised in rabbit), at 1/1000 in PBS-T with 2% NGS. After washing with PBS-T containing 2% NGS, the sections were incubated for 1 h with polyclonal swine anti-rabbit biotin conjugated antibody (Dako, Denmark) diluted 1/200 in PBS-T with 2% NGS at room temperature. Sections were washed again and incubated for 1 h in PBS-T containing the avidin-biotin complex (1/200; Vector Laboratories, USA). After washing in 0.05 M Tris-HCl, pH 7,6, the bound peroxidase was revealed using 0.0125% 3,3 diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich) and 0.025% H_2O_2 in the same buffer. Sections were mounted on gelatin-coated slides and dried at 37ºC during one overnight or at least 1 h, cleared in Xylene, mounted with Eukitt mounting medium and cover-slipped. The sections were observed in a light microscope

(Axioskop 40; Carl Zeiss, Germany), and images were acquired using a high resolution digital camera (Leica EC3 model) coupled to a computer with LAS 4.6.0. software (Leica Microsystems®). Light intensity, contrast and hue were maintained constant throughout image acquisition to ensure similar capture conditions across all sections.

Immunofluorescence

The slides containing LC were washed firstly in a 0.1M phosphate-buffered saline solution pH 7.4 (PBS). After inhibition of non-specific background with a solution of 1% borohydride in PBS and further washes in PBS and PBS-T (0.1 M PBS containing 0.3% Triton X-100), sections were incubated for 2 h in a blocking solution containing 0.1 M glycine and 10% normal goat serum (NGS) in PBS-T. Then, sections were incubated for 1 h at room temperature followed by 48 h at 4º C with a specific antibody against DBH (ImmunoStar, AB572229, anti-DBH raised in rabbit), at 1/1000 in PBS-T with 2% NGS. After this incubation period, slices were washed with PBS-T containing 2% NGS and incubated for 1h with polyclonal swine anti-rabbit biotin conjugated antibody (Dako, Denmark) diluted 1/200 in PBS-T with 2% NGS at room temperature. Further washes were made and the slides were then incubated for 1h in PBS-T containing streptavidine, Alexa Fluor® 594 conjugate (1/1000; Molecular probes). Finally, slices were washed in PBS-T and PBS and dried at 4ºC overnight. For analysis under the fluorescence microscope (Axio MRm, Zeiss, Germany) with the Axiovision 4.8. software (Carl Zeiss MicroImaging), slides were mounted with a glycerol solution prepared with PBS 0.4M.

7. Statistical Analysis

Statistical analyses were performed using the GraphPad Prism Software Version 6.0. Results were presented as the mean ± SEM. Behavioral data was analyzed by a two-way analysis of variance (ANOVA) followed by Sidak post hoc test for Von Frey test and by the Unpaired T-test for comparison between different groups in acetone test. Anxiety-depressive-like behavioral data was analyzed by Unpaired T-test for comparison between groups and Two-way ANOVA and Sidak post hoc for comparisons within the same group. For the experimental group 3 no statistical analyses were performed due to the low number of animals in each LV-Cre injected groups (see results). In all statistically analyses performed the level of significance was considered as $p<0.05$.

IV. Results

1. Evaluation of Anxiety and Depression in Neuropathic Pain Animals

Experimental group 1 (Scheme1) was designed in order to evaluate anxiety- and depression-like behaviors in a SNI model of neuropathic pain. For this goal, a group of SNI (n=5) and a group of sham (n=8) animals were tested 42 days after SNI or sham induction.

1.1. Anxiety- and depression-like behavior tests

The anxiety-like behavior tests (Figure 9 A, C, D) showed no significant differences between the SNI and sham animal groups. However, in the Open field (Figure 9A) and Elevated plus maze (Figure 9C) tests significant differences for comparisons within the same group were observed. Both SNI and sham groups showed a similar open field exploratory behavior, spending more time in an internal area when compared with the time spent in the center or in the periphery of the arena. The total open field travelled distance (Figure 9B) also did not show any significant difference between the SNI and sham animal groups, which excludes locomotor impairment. In the elevated plus maze test (Figure 9C) both SNI and sham animals showed a comparable behavior with a tendency to spend more time in the closed arms than in the open arms, with a significant difference being found in particular in the SNI group ($p<0.05$). In the MBT (Figure 9D), the SNI group buried a higher number of marbles. In all the tests assessing the anxiety-like behaviors there was a tendency for a more anxious behavior in the SNI group.

Unlike what was expected, in the FST (Figure 9E), sham animals had more propensity for depressive-like behavior than SNI, as they spent more time in immobility, but no significant differences were observed between groups.

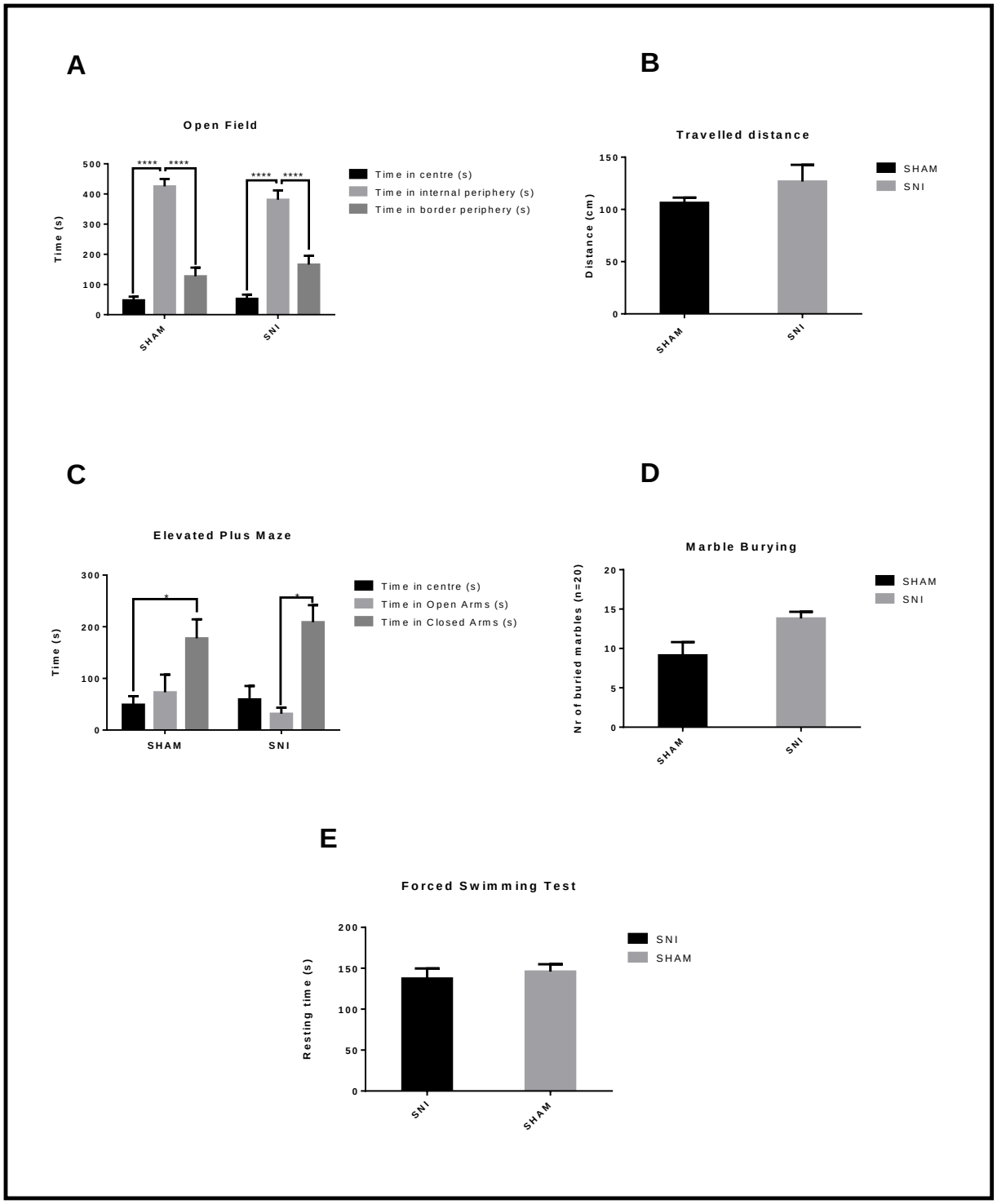


Figure 9- Results for anxiety- and depressive-like behavior tests in neuropathic pain animals. (A) OFT: Both groups spent more time in the periphery of the arena; (B) OFT: No significant differences were observed comparing SNI and sham groups in the locomotor activity; (C) EPM: Both groups spent more time in the close arms; (D) MBT: the SNI animals hid more marbles; (E) FST: the SNI group showed less immobility time (Unpaired T-test for comparison between the SNI and Sham groups; Two-way ANOVA and Sidak post hoc for comparisons within the same group; *p<0.05; ****p<0.0001).

1.2. Nociceptive behavior tests

After anxiety- and depression-like behaviors were tested, nociceptive tests were performed to confirm the hypersensitivity due to neuropathy in the same experimental group (Scheme1). In the von Frey (Figure 10A) a clear significant difference in pain tolerance between SNI and sham groups was verified. SNI mice showed lower tolerance for nociceptive stimuli by responding with more frequency to lower force monofilaments than the sham group.

The acetone test (Figure 10 B1 and B2) showed also significant differences between the SNI and sham control groups. Thus, the SNI group exhibited higher values in the acetone scale ($p<0.01$) and a longer paw withdrawal time ($p<0.05$), confirming the development of mechanical hypersensitivity and cold allodynia typically present in a neuropathic pain condition.

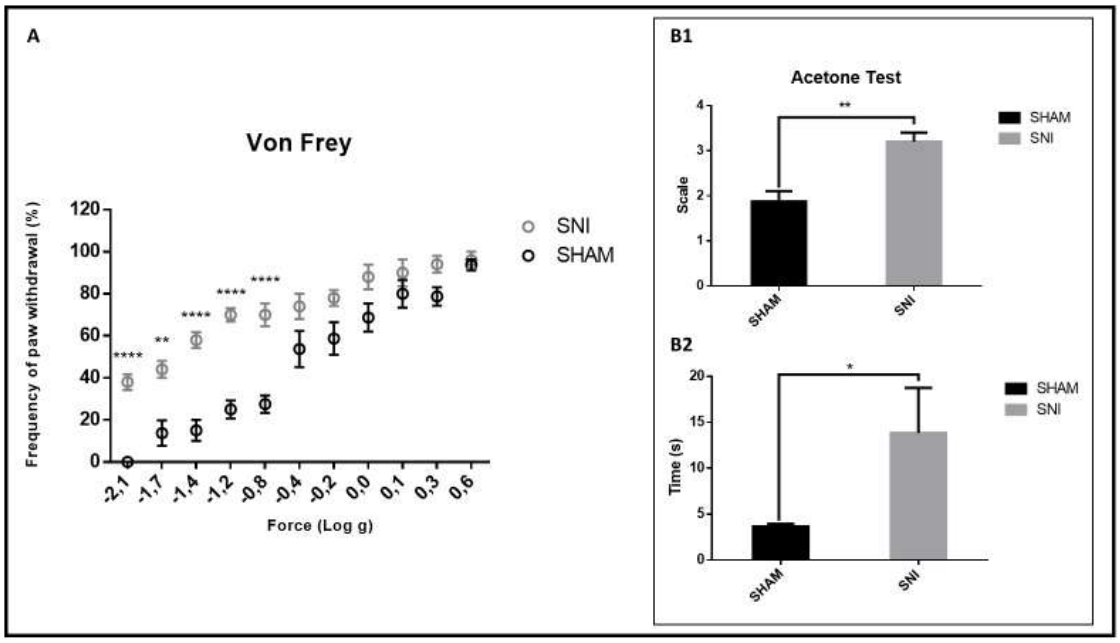


Figure 10-Results of nociceptive tests in neuropathic pain animals. (A) Von Frey test: frequency of paw withdrawal (Two-way ANOVA and Sidak post hoc; **p<0.01; ****p<0.0001) (B) Acetone test results for the SNI and Sham groups. (B1) Acetone scale scores; (B2) Time spent in paw withdrawal; (Unpaired T-test for comparison between SNI and Sham groups; *p<0.05; **p<0.01).

2. Effects of DBH ablation at the LC of conditional mice

2.1. Optimization of the experimental conditions

2.1.1. Optimization of stereotaxic coordinates for LV-Cre injection

In order to establish the optimal stereotaxic coordinates for LV-Cre injection, a sky-blue dye was injected bilaterally into the LC. To certify that the entire extension of the LC was covered by dye diffusion, two injections distanced by 100µm rostro caudally from each other were performed in the two lateromedial areas of the LC. Figure 11A shows an example of a stereotaxic injection at a rostral area of the LC while figure 11B demonstrates a second injection in a more caudal region.

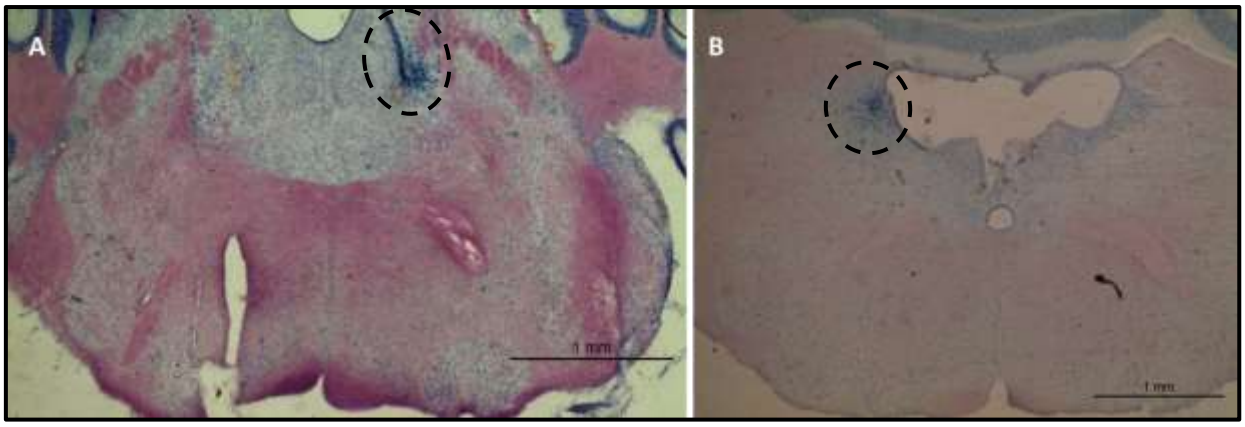


Figure 11- Optimization of stereotaxic injections into the LC. (A) Sky blue dye staining revealing the site of the 1st stereotaxic injection into the rostral LC (B) Sky blue dye staining revealing the site of the 2nd stereotaxic injection into the caudal LC. The injections are distanced 100 µm from each other.

2.1.2. Determination of the virus titer

Two different titers, 1x10^5 IV/mL (Figure 12A) and 1x10^6 IV/mL (Figure 12B), of LV-Cre were tested. LV-Cre also encodes a green fluorescent protein marker (EGFP; Scheme 4) which allows the detection of transduced neurons surrounding the injection site. Figures 12A and B show EGFP+ cells at 10 days after injection of each viral titer. A high number of EFGP+ cells as well as a higher diffusion capacity was observed at the concentration of 1x10^6 IV/mL (Figure 12 B) compared the lower concentration (Figure 12 A). This result revealed that injections of 400 nL of LV-Cre at a titer of 1x10^6 IV/mL are the best to ensure a satisfactory lentiviral transduction.

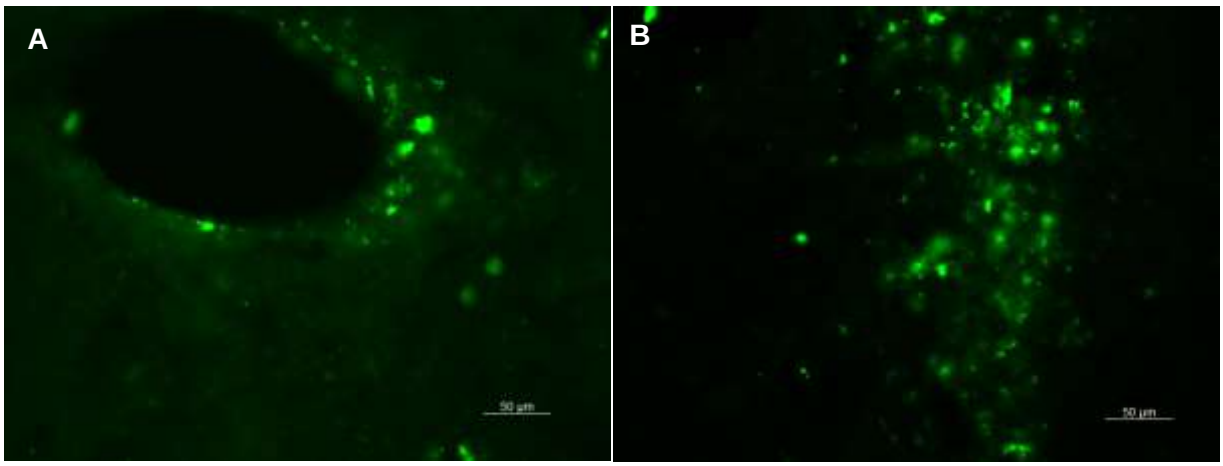


Figure 12- Stereotaxic injections of LV-Cre at two different concentrations. (A) Stereotaxic injection with LV13: hSyn-eGFP-Cre (Gene Vector and Virus Core, Stanford University, USA), at a concentration of 1x10^5 IV/mL, showing low EGFP expression; (B) Stereotaxic injection with LV13: hSyn-eGFP-Cre (Gene Vector and Virus Core, Stanford University, USA), at a concentration of 1x10^6 IV/mL, showing a higher number of cells expressing EGFP, which indicates a more efficient lentiviral transduction and also a better diffusion of the virus.

2.1.3. Verification of *Dbh* gene ablation by LV-Cre

In order to verify whether LV-Cre can deplete the *Dbh* gene after a minimum time of 10 days post-stereotaxic injection, we assessed the DBH expression by performing immunohistochemistry in brain sections containing the LC at the optimized stereotaxic coordinates. Immunolabelling against DBH showed almost complete ablation at the injection site (right LC; Figure 13).

Considering that in the literature[82] a period of 10 to 14 days after virus injections is recommended to ensure efficient gene expression levels, a period of 14 days after injection was chosen to guarantee an effective ablation of the *Dbh* gene.

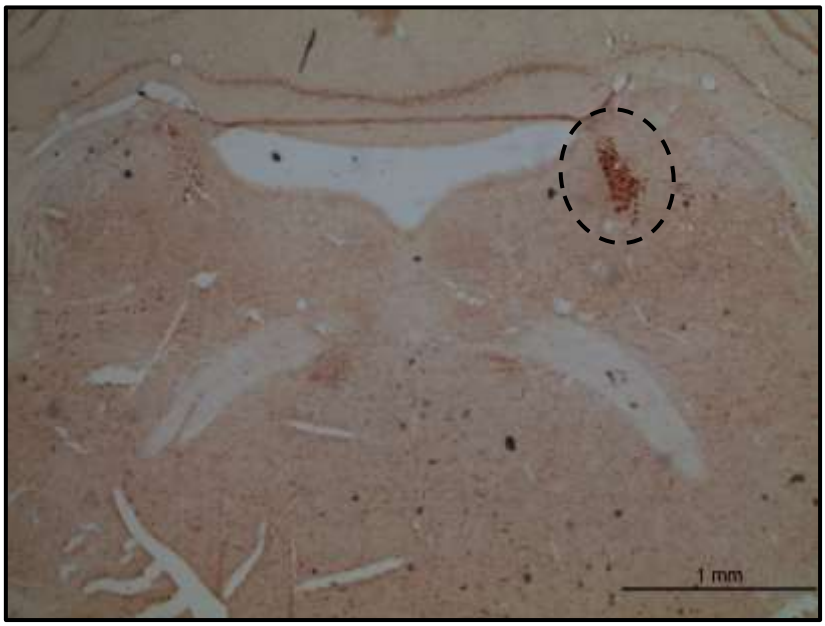


Figure 13- Immunoreaction against DBH revealed with DAB (brown) in the LC 10 days post stereotaxic injection of LV-Cre. The right LC showed almost complete ablation of the *Dbh* gene compared with the left LC (circumference).

2.2. Characterization of Dbh ablation at the injections sites of the conditional mutant mice

For the characterization of *Dbh* ablation, animals from the experimental group 3 (Figure 14) composed by 8 conditional mutant mice injected with LV-Cre at LC were sacrificed and immunolabeled against DBH. The injection sites and *Dbh* ablation were posteriorly identified. Figure 14A-H (A-rostral → H-caudal) depicts LV-Cre injections sites in diagrams of the mice brains of all animals. Two of the total number of animals were correctly injected into the LC at the levels -5.40 and -5.68 (Figure 14 C and E, represented with ✚ and ■). Injections at these coordinates will have the designation *LV-Cre-center LC*. Two other animals had the injections localized in a more caudal region of the LC, namely at levels -5.68 and -5.80 (Figure 14 E, represented by ● and ⬬), now designated by *LV-Cre-caudal LC*. One animal had the injection in the rostral LC, at level -5.34 (Figure 14, represented by ▲), now designated by *LV-Cre-rostral LC*. The remaining three animals did not show significant ablation of *Dbh* gene expression at the LC nor EGFP signal in the vicinity, indicating that the injection sites were off target (Figure 14, represented by , ▲ and ⧈).

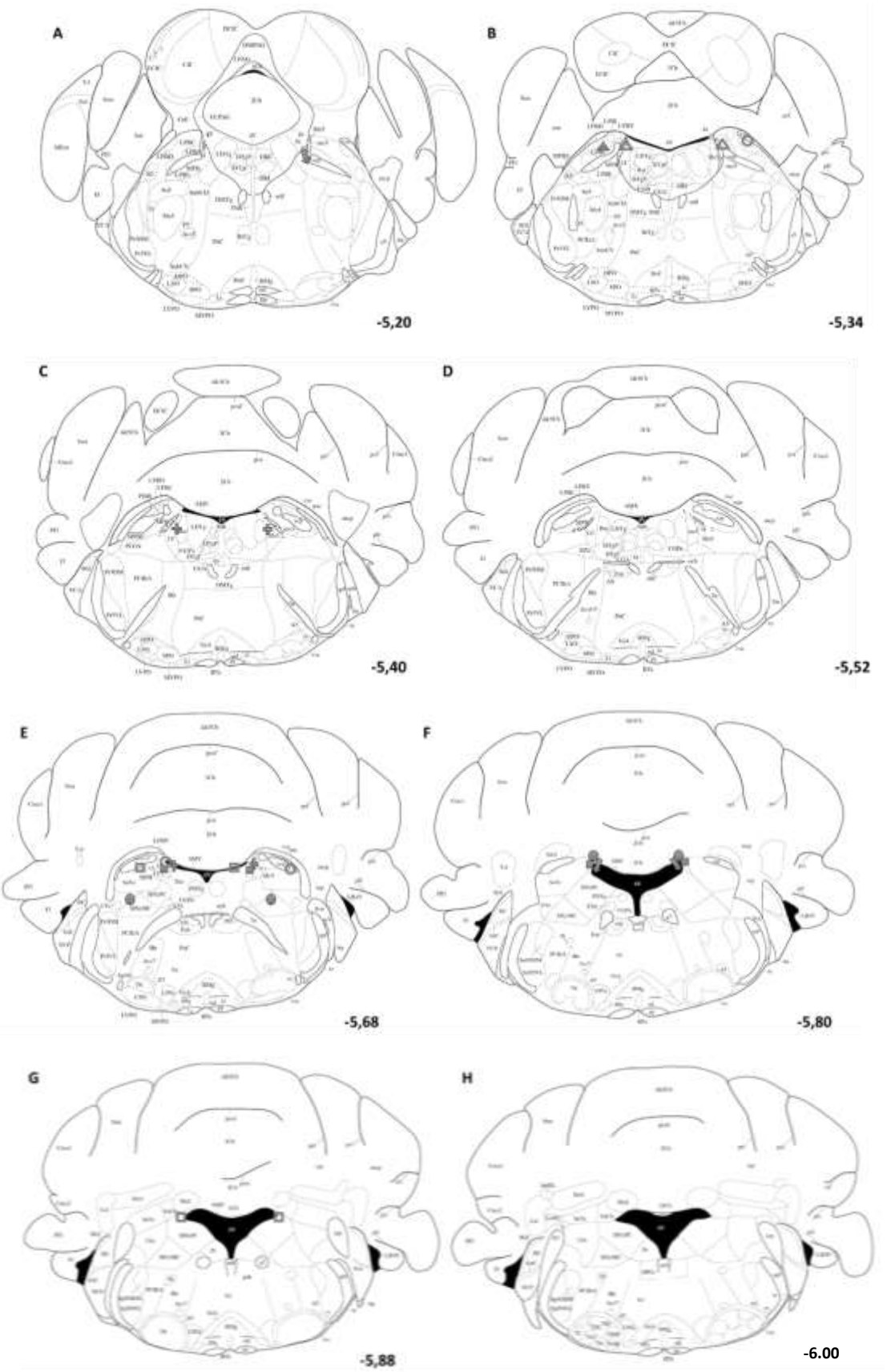


Figure 14- Diagrams depicting the LV-Cre injections sites. (A-H) Represent the different rostro-caudal levels of the LC and adjacent levels of the brain. The numbers on the right indicate the Bregma level of each section. ✜ and ■ represent injections at the levels -5.40 and -5.68 of the LC, designated as LV-Cre-center LC; ● and ◉ represent

injections at the more caudal levels -5.68 and -5.80, designated as LV-Cre-caudal LC; ▲ represents injections at the more rostral level -5.34, designated as LV-Cre-rostral LC. Symbols ▼ , △ and ▣ represent injections not targeting the LC.

Animals with LV-Cre injections correctly placed at the LC showed a significant level of DBH ablation but not a total ablation. The ablation of DBH in transduced cells was total as shown by the absence of co-localization of EFGP with DBH (Figure 15). However, surrounding the injection sites, a small percentage of DBH+ cells was not transduced, as indicated by no EGFP labelling (Figure 15) which indicates that a few DBH+ neurons remained intact and continued to produce DBH.

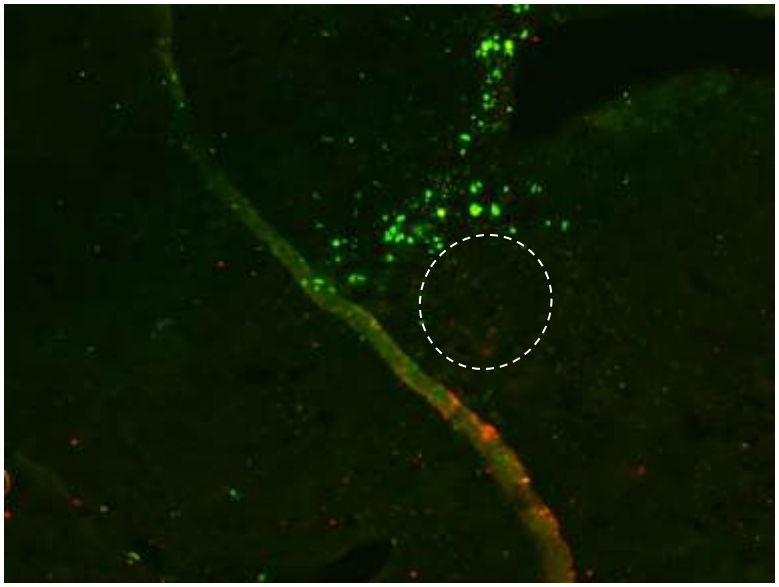


Figure 15- Immunofluorescence against DBH. Fluorescent microscope photo images depicting an immunoreaction against DBH (red, labelling the neuronal cell bodies) and the green fluorescence from transduced neurons (green, labelling at the injection site) in the LC. The areas surrounded by white dashed lines show a small percentage of DBH cells no transduced.

2.2.1. Nociceptive behavior

2.2.1.1. Effect of the stereotaxic injection before SNI induction

To evaluate if the injection procedure *per se* could affect the nociceptive behavior of the animals, von Frey tests were performed before (Baseline 1; Scheme 3) and 13 after LV-Cre injection (Baseline 2; Scheme 3). No significant differences were observed when comparing the animals before or 13 days after LV-Cre injection. This result also shows that at 13 days after LV-Cre, an almost total or partial ablation of *Dbh* does not have an impact on basal mechanical sensitivity of naive animals.

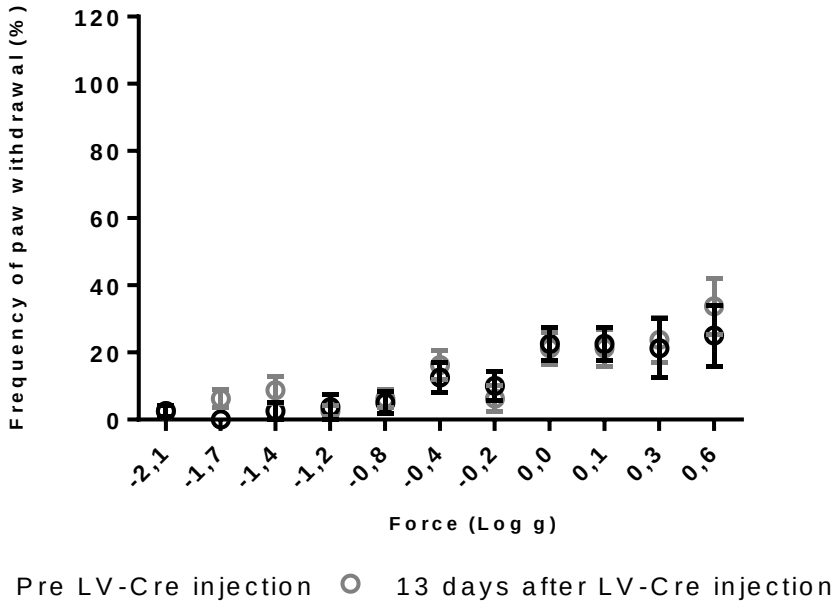


Figure 16- Frequency of paw withdrawal in response to Von Frey filaments of the animals before and after LV-Cre injection. No significant differences were observed.

2.2.1.2. Time course effects of Dbh ablation at the LC

The effects of *Dbh* ablation on the development and maintenance of pain behaviors after SNI induction (Scheme 3) were analyzed according to the different rostro-caudal levels of the LC where DBH ablation occurred (*LV-Cre-center LC: n=2; LV-Cre-caudal LC: n=2; LV-Cre-rostral LC: n=1*; Figure 14) and compared to mice from experimental group 2 (SNI animals not subjected to stereotaxic LV-Cre injections n= 5; scheme 2).

Von Frey test behavior

No statistical analyses were performed, considering the small number of animals of LV-Cre injected animals for each targeted LC region. At baseline (i.e. before SNI induction), control and LV-Cre-rostral-injected animals showed an increase of frequency of paw withdrawals, in response to von Frey filaments, proportional to the increasing forces applied to the paw, unlike *LV-Cre-center*- and LV-Cre-caudal-injected animals which showed very low frequency of paw withdrawals to all forces applied (Figure 17A). After SNI induction and at all times tested, control animals, maintained a high frequency of paw withdrawals to the higher forces applied to the paw, with in addition a clear upward shift in frequency responses to the lower forces applied (Figure 17B-E) which demonstrates the development of mechanical allodynia.

The most apparent differences between the experimental groups seem to be more evident at day 7 and 14 (corresponding to initial timings of development of the SNI neuropathy) with *LV-Cre-center*- and *LV-Cre-caudal*-injected animals responding in general with lower frequency rates at all von Frey filaments applied compared to control and LV-Cre-rostral-injected animals (downward shift; Figure 17B and C). At day 21, this downward shift is only apparent for the frequency of withdrawals in response to the higher forces applied (Figure 17 D).

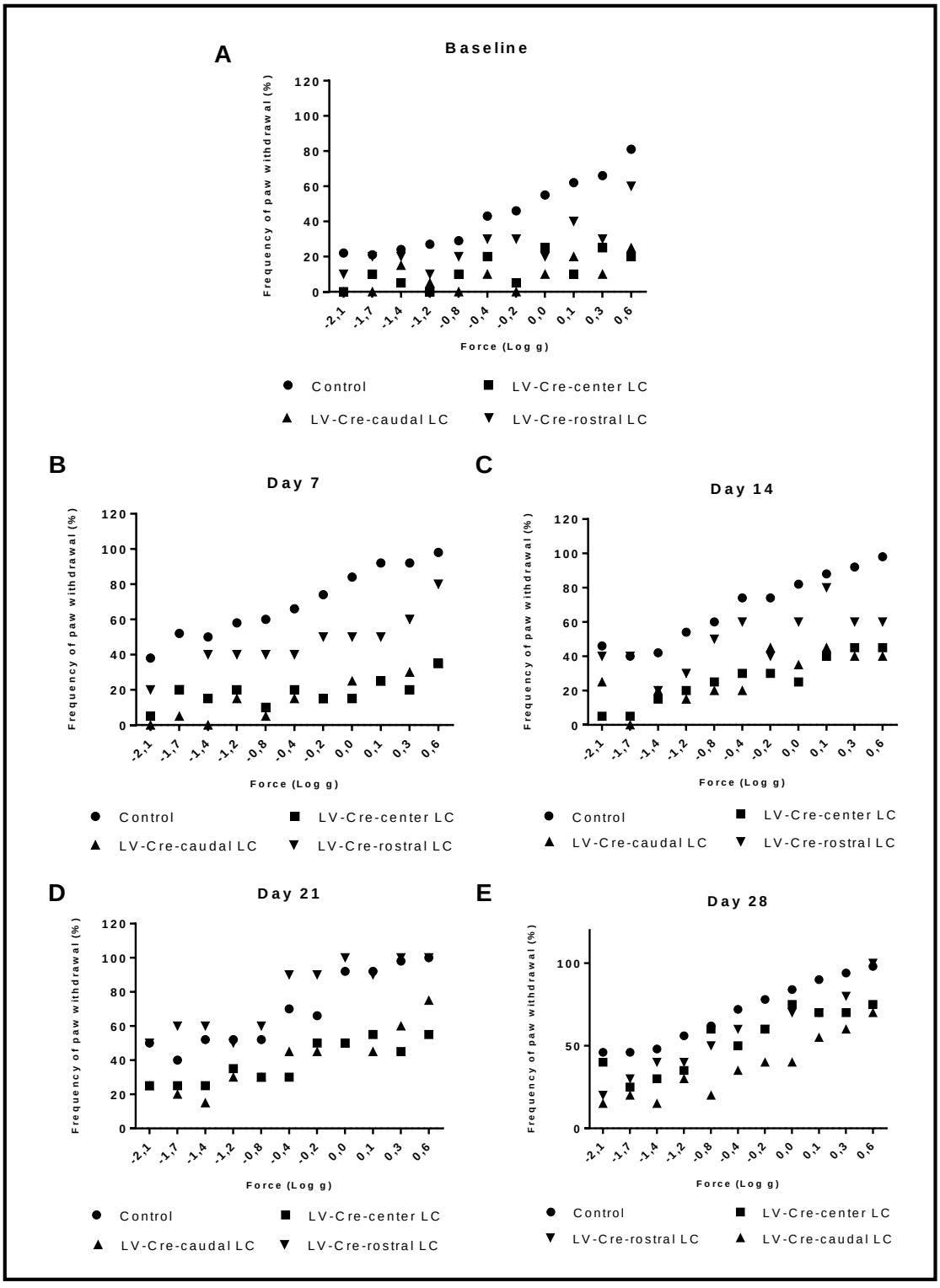


Figure 17- Frequency of paw withdrawal to von Frey monofilaments. (A) Baseline 2 taken before SNI induction i.e. 13 days after LV-Cre injection; (B) 7 days after SNI induction; (C) 14 days after SNI induction; (D) 21 days after SNI induction and (D) 28 days after SNI induction. LV-Cre-injected animals analyzed (*LV-Cre-center LC:* n=2*; LV-Cre-caudal LC:* n=2*; LV-Cre-rostral LC:* n=1*);* Control group n= 5.

Acetone test behavior

As expected, the control group showed higher values in the acetone scale and paw withdrawal latency times throughout disease development than at baseline, which were more evident at 7 days and particularly at 28 days of SNI (Figure 18 A-J). At baseline, (i.e. before SNI induction) all LV-Cre injected mice showed lower values in the acetone scale (Figure 18 A) and lower paw withdrawal latency times (Figure 18 B) than controls, indicating a reduced cold allodynia. This tendency was maintained on day 7 of SNI, except for the animals from the LV-Cre-rostral LC group, which showed higher values in the acetone scale (Figure 18 C) and higher paw withdrawal latency times (Figure 18 D), similar to control values, suggesting that they exhibit an increased cold allodynia, compared to the other LV-Cre injected groups. From day 7 onwards until day 21, the difference between the groups was attenuated except for the LV-Cre-caudal LC, which continued to show lower values in the acetone scale and lower paw withdrawal latency times than the other experimental groups. In fact, the LV-Cre-caudal LC group showed identical values in the acetone scale (Figure 18 A and C) and presented lower paw withdrawal latency times (Figure 18 B and D) at day 7 when compared with baseline. In contrast, the LV-Cre-rostral LC group presented acetone scale values and paw withdrawal latency times similar to those of the control group, except at 28 days of SNI, when a decrease was observed.

These results seemed to confirm the development of cold allodynia typically present in a neuropathic pain condition for all groups except for the LV-Cre-caudal group demonstrated a lower sensitivity to cold stimuli.

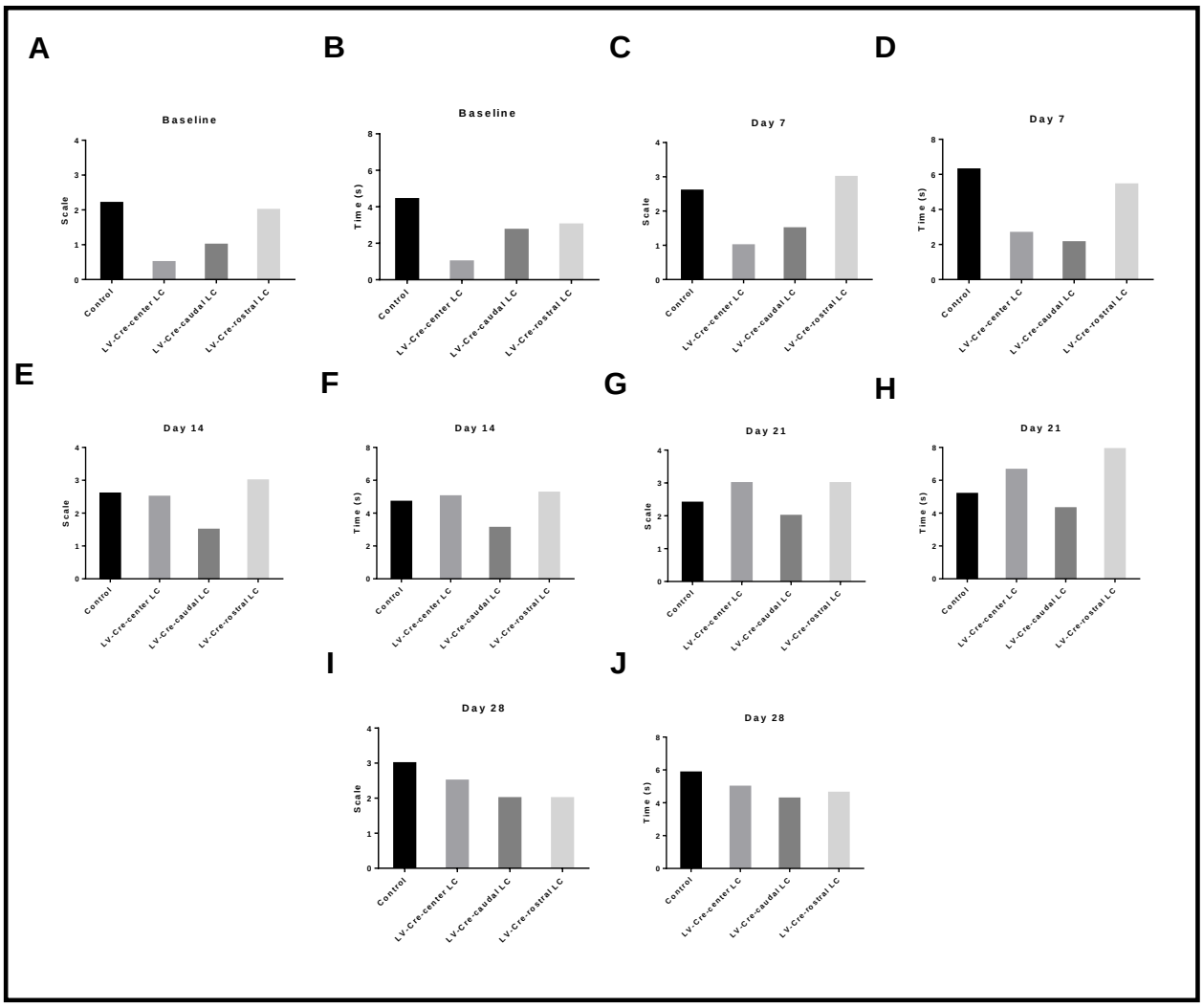


Figure 18- Acetone test. (A, C, E, G, I) Acetone scale scores at baseline (before SNI induction) and on days 7, 14, 21 and 28 respectively; (B, D, F, H, J) Time spent in paw withdrawal at baseline (before SNI induction) and on days 7, 14, 21 and 28 respectively. LV-Cre-injected animals analyzed (*LV-Cre-center LC:* n=2*; LV-Cre-caudal LC:* n=2*; LV-Cre-rostral LC:* n=1*);* Control group n= 5.

2.2.2. Anxiety- and depression-like behaviors

Although no statistical analyses were performed considering the small number of animals of each LV-Cre groups, data from the Open Field test for anxiety-like behavior showed that the LV-Cre-center and LV-Cre-caudal LC groups had a tendency to explore more the border periphery of the arena, spending more time in this area than the control and LV-Cre-rostral groups which appeared to prefer the internal periphery area (Figure 19 A). The travelled distance of the LV-Cre-center and LV-Cre-caudal LC groups was increased when compared with the other groups (Figure 19 B). In the Elevated plus maze test (Figure 19 C), all groups showed a tendency to spend more time in the closed arms except the LV-Cre-rostral LC group which remained a larger amount of time in the opened arms. Both results seem to indicate that the LV-Cre-rostral LC animals exhibit less pronounced anxiety-like behavior. In the Marble Burying test (Figure 19 D) no obvious differences between groups could be observed, though the control and LV-Cre-rostral LC groups buried more marbles than the other groups.

In the FST (Figure 19 E), the LV-Cre-center and LV-Cre-rostral animals had more inclination for spending more time in immobility than the LV-Cre-caudal and LV-Cre-rostral groups, indicating more pronounced depressive-like behaviors, although only smaller differences were observed.

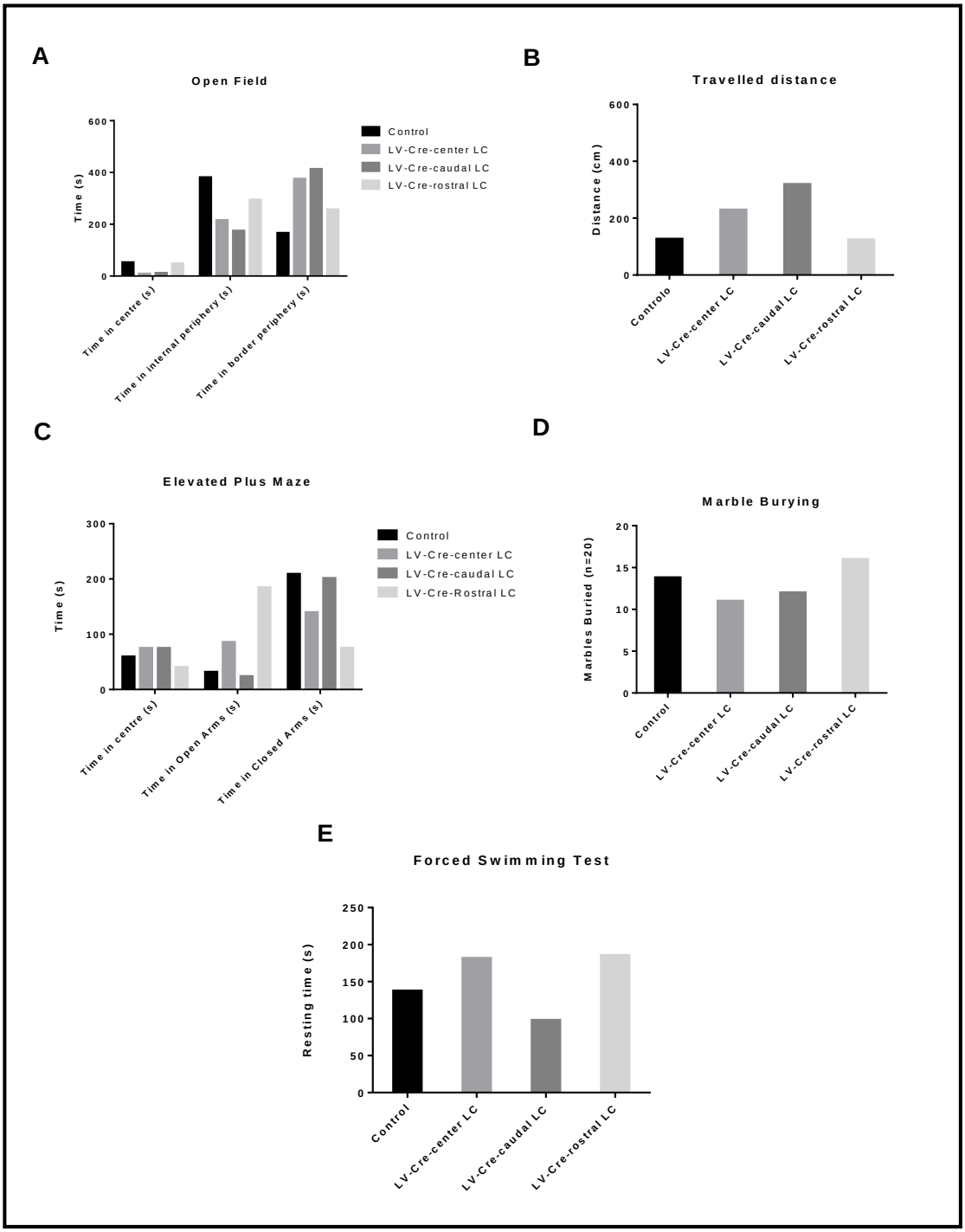


Figure 19-Results for the anxiety and depressive-like behavior tests in SNI animals injected with LV-Cre and SNI animals without LV-Cre injection (control). (A) OFT: the LV-Cre-caudal LC groups had tendency to explore the border periphery while the LV-Cre-rostral groups appeared to prefer an intermediated area; (B) OFT locomotor activity: the travelled distance of the LV-Cre-center and LV-Cre-caudal LC groups was higher than in the other groups; (C) EPM: the LV-Cre-rostral LC group remained a larger amount of time in the opened arms;(D) MBT: No obvious differences between groups were observed although LV-Cre-rostral LC and control groups had buried a few more marbles than the other groups;(E) FST: the LV-Cre-center and LV-Cre-rostral animals showed higher immobility time than the LV-Cre-caudal and LV-Cre-rostral groups. LV-Cre-injected animals analyzed (*LV-Cre-center LC:* n=2*; LV-Cre-caudal LC:* n=2*; LV-Cre-rostral LC:* n=1*);* Control group n= 5.

V. Discussion

It has been recognized that the experience of pain is highly variable between individuals. Pain is the result of an activation of sensory receptors specialized to detect actual or impending tissue damage. However, a direct correlation between activation of nociceptors and the sensory experience of pain is not always apparent. Even in cases in which the severity of injury appears similar, individual experiences may differ dramatically. Many factors as the emotional state, degree of anxiety, attention and distraction, past experiences, memories and others can either enhance or diminish the pain experience[12].

The degree of pain perceived in response to a given noxious stimulus is significantly influenced by the context within which the injury is suffered[108]. This variation is due to endogenous analgesic mechanisms within the central nervous system that act to modulate pain. Reynolds, in 1969[109], reported that electrical stimulation of midbrain produces profound analgesia and since then, increased attention has been focused on descending control systems which can influence the spinal transmission of noxious inputs[110]. The noradrenaline-containing neurons of the LC, A_5 and A_7 cell groups have been implicated as key components of the descending control system [25, 111]. Given that, the LC is proposed to play an important role in an endogenous descending pain control circuit[110, 111] and the noradrenergic circuit component seems to be functionally underactive in chronic neuropathic pain, revealing an interesting strategy to be explored.

The main goal of this project was to study the effects of the deletion of the *Dbh* gene on the induction and development of neuropathic pain by genetically manipulation of the noradrenergic system in mutant mice. For this purpose, *Dbh* flox$^{+/+}$ mice were used to establish a protocol for *Dbh* gene ablation by stereotaxic injection into the LC of LV-Cre, a lentiviral vector expressing the Cre recombinase enzyme, which deletes the loxP flanked *Dbh* gene.

In the past decade, many research groups succeeded in observing anxiety or depression related behaviors in models of sciatic nerve injury[52, 112-115], as well as anxiety-like behavior in models of HIV and antiretroviral-induced neuropathy[115] and in a model of postherpetic neuralgia[53, 116]. Having these results in consideration, the first aim of this study was to understand some of the mechanisms underlying the anxiodepressive comorbidity in a model of chronic neuropathic pain. In order to evaluate anxiety and depression in neuropathic pain animals, SNI and sham surgeries were performed in Dbh $^{flox+/-}$ or Dbh $^{flox-/-}$ animals. Anxiety and depression-like behavior tests were performed 42 days after surgery and at day 49 the nociceptive behaviors were analyzed to confirm the

development of hypersensitivity to mechanical and thermal stimuli, typically present during a peripheral neuropathy. The results showed no significant differences between the SNI and Sham animal groups in what concerns the anxiety- and depressive-like behaviors; however, in the open field and Elevated Plus Maze tests significant differences for comparisons within the same group were observed within both groups. Both groups showed a similar open field exploratory behavior, spending more time in internal area when compared with the time spent in the center or in the periphery of the arena. Although, some authors defend that animal models submitted to a traumatic lesions appear to travel less distance when compared with sham animals[117], no significant differences were observed in Open Field locomotor activity, which excludes any locomotor impairment due to pain model induction. The Elevated Plus Maze showed a comparable behavioral pattern between groups with animals revealing a tendency to spend more time in the closed arms than in open arms, and this difference was statistically significant in the SNI group ($p<0.05$). The parameter analyzed in this test is the time spent in the different arms of the platform (open, closed and center arms), with the objective of exposing the animals to aversive conditions as height and open spaces. The fact that the animals prefer the closed arms instead of exploring the open arms is indicative of anxiety-like behavior[118], while the time spent in the center can indicate an incapacity on decision-making. The differences observed seem to be in accordance with previously published studies, as described by Paterson and collaborators (2010)[119]. On the Marble Burying test, the SNI group buried a higher number of marbles, although differences were no statistically significant in comparison to sham mice. The reflection created by the light over the marbles causes an aversive stimulus inside the cage leading the animal to bury the marbles, a behavior that is associated with the manifestation of anxiety, as described by De Boer and Koolhaas (2003) [120]. Thus, in all the tests assessing the anxiety-like behaviors there was a tendency for a more anxious behavior in the SNI group of animals at 6 weeks of disease duration. Other authors observed shorter times for developing anxiety, but there is some controversy associated with the beginning of this condition. Also, any environmental conditions as well as the physical state of the animals may influence the outcome of anxiety and depressive-like behavioral tests, which can account for the lack of statistical significant differences between the sham and SNI groups, although these parameters have been thoroughly controlled throughout experimentation.

To evaluate the depressive-like behavior, the forced swimming test was performed. The time spent in immobility (*i.e.*floating with the absence of any movement except for those necessary for keeping the nose above water) was the parameter measured considering

the threat of drowning[121]. When submitted to this situation animals have two options, fight for survival or remain immobile, the last option being an indication of resignation associated to depressive conditions[122, 123]. Unlike what was expected, in FST, sham animals had more propensity for depressive-like behaviors than SNI, as they spent more time in immobility, but no significant differences were observed between groups. This result can be explained with the studies developed by Yalcin et al. (2011) which observed that depressive-like behaviors associated with traumatic injuries appear at later time points, namely after 8 weeks[114]. In contrast, there are some publications reporting the start of depressive-like behaviors at 4 weeks after SNI induction[124, 125].

To confirm the development of hypersensitivity due to neuropathy the von Frey and acetone tests were also performed. In the Von Frey test, SNI mice showed lower tolerance for mechanical stimuli by responding with more frequency to lower force monofilaments, which indicates that they develop lower paw withdrawal thresholds than the sham group, confirming then a successful induction of the neuropathy. These results appear to be in accordance with the literature [99, 126] , which report the presence of allodynia 3 days after model induction. The experimental group was analyzed at day 49, precisely to confirm that SNI induction caused neuropathic pain symptoms. The existence of cold allodynia was also confirmed with significant differences being detected between the SNI and sham groups. Thus, the SNI group exhibited higher values in the acetone scale and a longer paw withdrawal latency. The development of mechanical hypersensitivity and cold allodynia in SNI animals, which is corroborated by the studies of Decosterd and Woolf (2000)[79].

Although the LC sends projections throughout the neuraxis, most pain studies have concentrated upon the axonal projections to the spinal dorsal horn. These release noradrenaline [127, 128] that acts via α_2-AR to inhibit both primary afferents and second order projection neurons[129-134]. Electrical or chemical stimulation of the LC has been shown to be analgesic in a number of acute pain models[111, 132, 133]. Additionally, in chronic neuropathic pain models, there are plastic changes in the noradrenergic innervation of the spinal dorsal horn [135] and increases in α_2-AR sensitivity[136, 137]. Our investigations focused on the genetic manipulation of the LC by promoting the ablation of Dbh in mutant mice that underwent a stereotaxic injection for delivery into the LC of a lentivirus expressing the Cre recombinase enzyme (LV-Cre) in order to induce a conditional Dbh ablation. After a hard optimization of the experimental conditions, comprising the establishment of stereotaxic coordinates for LV-Cre injections into the LC and the determination of the virus titer to inject, we used an experimental group of animals Dbh $^{flox+/+-}$ for the evaluation of the efficacy of conditional *Dbh* ablation at the LC of the mice.

The same mice were used to evaluate the effects of NA depletion on the nociceptive and anxiety/depression-like behaviors during a neuropathic pain condition. The first results showed a heterogeneous ablation in different rostro-caudal extensions of the LC. In fact, we were not able to establish with precision the best stereotaxic surgical procedure for total ablation of Dbh. However, a partial and almost total ablation was achieved and the main goal of our investigations has become the evaluation of the effects on the nociceptive and anxiety-/depression-like behaviors of Dbh ablation at different rostro-caudal regions of the LC. After the characterization of the efficacy of Dbh ablation at the injection sites of the conditional mutant mice, animals were divided in three groups according to the site of injection and the LC levels affected. The injections correctly localized at LC levels -5.40 and -5.68 were grouped under the designation *LV-Cre-center LC*. The injections localized in a more caudal region of the LC, namely at levels -5.68 and -5.80, were classified as *LV-Cre-caudal LC*, while the animal with the injection in the rostral LC, at level -5.34, was sorted as *LV-Cre-rostral LC.* With this division, each group of animals became reduced which did not allow any reliable statistical analysis.

Before the evaluation of the time course effects of Dbh ablation at the LC it was verified if the stereotaxic injection *per se* could affect the nociceptive behavior of the animals. For that purpose, von Frey tests were performed before and after LV-Cre injection, before inducing SNI, and no significant differences were observed, regardless of the fact that the animals had almost total or partial ablation of Dbh expression or not. These data indicate the LV-Cre injection procedure per se has no effects on mechanical sensitivity in non-neuropathic pain conditions. Therefore, the procedure is appropriate to study the effects of the noradrenergic system in neuropathic pain development and maintenance.

The effects of Dbh ablation, and therefore of NA neurotransmission impairment, at different rostro caudal-levels of the LC on the nociceptive behavior during neuropathic pain development and maintenance were analyzed by the von Frey and acetone tests. The frequency of paw withdrawal of the animals in response to von Frey filaments increased with the intensity of the force applied in all experimental groups. However, the frequency of responses on day 7 and day 14 of SNI was different between groups showing Dbh ablation at different rostro-caudal extensions of the LC. The control and *LV-Cre-rostral LC* groups responded with higher frequency to von Frey filaments when compared with the *LV-Cre-center LC* and *LV-Cre-caudal* groups. However, on day 7 the *LV-Cre rostral LC* group showed lower frequency of responses than the control mice, particularly evident for higher force monofilaments. After day 14 the differences in the frequency of responses between groups were minimized throughout the time of SNI development.

In the acetone test, the control groups showed higher values in the acetone scale and paw withdrawal latency times throughout disease development than at baseline, which were more evident at 7 days and particularly at 28 days of SNI. These results are in accordance with what was expectable. Interesting data were obtained at baseline, before SNI induction for all LV-Cre injected mice, which showed lower values in the acetone scale and lower paw withdrawal latency times than controls indicating a reduced cold allodynia. This tendency was maintained on day 7 of SNI, except for the animals from the *LV-Cre-rostral group*, which showed higher values in the acetone scale and high paw withdrawal latency times, similar to the control values. These results suggest that *LV-Cre-rostral group* exhibit an increased cold allodynia, compared to the other LV-Cre injected groups. These differences between groups were attenuated from day 7 onwards until day 21, except for the *LV-Cre-caudal LC*, which continued to show lower values in the acetone scale and lower paw withdrawal than the other groups. Data confirm the development of cold allodynia, which is typically present in a neuropathic pain condition for all groups except for the LV-Cre-caudal group, which demonstrated a lower sensitivity to cold stimuli.

The values in the acetone scale and paw withdrawal latency times lower than in controls, indicating the presence of cold allodynia at baseline, and the fact that the control and *LV-Cre-rostral LC* groups responded with higher frequency to von Frey filaments when compared with *LV-Cre-center LC* and *LV-Cre-caudal* groups appears to counter our expectation. Although the LC is predominantly associated with pain inhibition, our results suggest that it may also play a role in the facilitation of chronic pain. This pronociceptive effect, particularly evident in *LV-Cre-center LC* and *LV-Cre-caudal LC*, seems to reinforce the reports of Brightwell and Taylor (2009) which showed that the LC facilitates the maintenance of neuropathic pain[138]. Similarly, Martins et al. (2010) reported a facilitatory effect of the LC on neuropathic pain mediated via the medullary dorsal reticular nucleus[42]. Additionally, a rostro-caudal differentiation in the LC role in nociception appears to occur, as NA synthesis depletion in the rostral LC does not have a similar effect on the nociceptive behavior.

The anxiety and depression-like behaviors observed on the open field test showed that the *LV-Cre-center* and *LV-Cre-caudal LC* groups had a tendency to explore more the border periphery of the arena, spending more time in this area than the control and *LV-Cre-rostral LC* groups, which appeared to prefer the internal periphery area. On the Elevated Plus Maze, all groups showed a tendency to spend more time in the closed arms except the *LV-Cre-rostral LC* group which remained a longer amount of time in the open arms. Both results seem to indicate that the *LV-Cre-rostral LC* animals exhibit a

less pronounced anxiety-like behavior. On the opposite, in the MB behavior the control and *LV-Cre-rostral LC* group buried more marbles than the other groups. Additionally, in the FST, the *LV-Cre-center* and *LV-Cre-rostral* animals had more inclination for spending more time in immobility than the *LV-Cre-caudal LC* and *LV-Cre-rostral LC* groups, indicating more pronounced depressive-like behaviors in the first two groups of mice.

The difference between the nociceptive and anxiety-depressive-like behaviors observed between the LV-Cre groups suggests that different areas of the LC may be correlated with different nociceptive and affective components. Indeed, we observed that more caudally located modifications on the synthesis of NA in the LC (*LV-Cre-caudal LC*) leads to a pronociceptive effect while more rostral changes in LC the NA system *(LV-Cre-rostral LC)* seems to have implications in the anxiety and depression-like behaviors, suggesting that the rostral LC may be correlated with the affective component of pain perception. Interestingly, this is a topic of current research in pain neurobiology. Indeed, in a recent study, published in October 2017, the authors expressed chemogenic actuators selectively in LC neurons with spinal or prefrontal cortex projections by using viral vectors. With this methodological approach they saw that the activation of the LC neurons with spinal projections produced robust, lateralized anti-nociception while the activation of LC neurons with prefrontal cortex projections produced aversion. They also demonstrated that in a neuropathic pain model the activation of spinal projections reduced the hind-limb sensitization and induced conditioned place preference for conditions where their pain is alleviated. By contrast, activation of pre-frontal projections exacerbated the spontaneous pain, produced aversion and increased the anxiety-like behavior. This independent, contrasting modulation of pain-related behaviors mediated by distinct noradrenergic neuronal populations provides evidence for a modular functional organization of the LC[139], as our preliminary studies also suggest.

VI. Conclusions and future perspectives

In this work we conclude that:

a) Anxiety- and depression-like tests in SNI (neuropathic pain condition) and sham animals (Dbh $^{flox+/-}$ or Dbh $^{flox-/-}$) showed:

- Similar open field exploratory behaviors
- Comparable behavior in the Elevated Plus Maze with a tendency to spend more time in the closed arms than in open arms.
- On Marble Burying test the SNI group buried a higher number of marbles.
- No significant differences were observed in the depressive-like behaviors.

b) Nociceptive behavioral tests in SNI (neuropathic pain condition) and sham animals (Dbh $^{flox+/-}$ or Dbh $^{flox-/-}$) confirmed the development of mechanical and thermal hypersensitivity due to neuropathy. The injection procedure in the LC *per se* did not affect the development of this hypersensitivity due to the pain condition.

c) After stereotaxic injection with LV-Cre (hSyn-eGFP-Cre):

- Heterogeneous ablations in different areas of the LC were observed.
- The difference between nociceptive and anxiety-depressive-like behaviors observed for the different rostro-caudal extensions of LC targeted by LV-Cre injections suggests that different areas of the LC may be correlated with different nociceptive and affective components of pain perception.

Thus, our future perspectives aim at enhancing the number of animals with partial or total Dbh ablation at different rostro-caudal levels of the LC and determine the effects of Dbh KO on:

a) The induction and development of neuropathic pain, allowing us to study the consequences of manipulating the noradrenergic system at the LC on the nociceptive responses and to determine if noradrenergic pain modulation depends on different stimulus modalities. With this we hope to establish possible different functional associations between the specific rostro-caudal areas of the LC and nociception;

b) The development of depressive and anxiety-like behaviours during neuropathic pain; with this we hope to establish possible different functional associations between the specific rostro-caudal areas of the LC and anxiety and depressive-like behaviors.

c) ERK1/2 activation in supraspinal areas connected to the LC related to nociceptive and anxiety and depression-like behaviours;

d) The neurochemical profile of neurotransmitter release in supraspinal areas connected to the LC due to Dbh KO through microdialysis, where it is intended to uncover: (1) alterations of neurotransmitter release, in the chosen areas, induced by neuropathic pain; (2) how the noradrenergic modulation of these regions is affected by neuropathic pain; (3) and how the depletion of NA input affects neurotransmitter release. Neurochemical analyses will be conducted at the timing when Dbh depletion induces significant behavioural alterations;

VII. Appendix

Composition of solutions:

1. PHOSPHATE BUFFER SALINE (PBS) (1 L)

Phosphate buffer (PB) 0.1 M pH = 7.2:

Na2H2PO4H2O – 15.60 g

K2HPO4 – 17.4 g

H2O up to 1L

PBS:

PB 250 ml

H2O up to 1 L

NaCl – 9 g

2. PHOSPHATE BUFFER SALINE WITH TRITON X-100 (PBS-T)

PBS – 996 mL

Triton X-100 – 4 mL

3. TYRODE'S SOLUTION (1 L)

NaCl – 6.8 g

KCl – 0.40 g

MgCl2.6H2O – 0.32 g

MgSO4.7H2O – 0.1 g

NaH2(PO4).H2O – 0.17 g

Glucose 1 g

NaHCO3 – 2.2 g

H2O up to 1 L

4. CRYOPROTECTOR SOLUTION (1 L)

PB 0.1 M pH = 7.2 – 125 mL

H2O – 375 mL

Sucrose – 300 g

Ethylene glycol – 300 mL

PB 0.1 M pH = 7.2 up to 1 L

VIII. References

1. van Griensven H, Strong J, Unruh AM. *Pain E-Book: A Textbook for Therapists*: Elsevier Health Sciences; 2013.

2. Cohen SP, Mao J. Neuropathic pain: mechanisms and their clinical implications. *BMJ : British Medical Journal.* 2014;348.

3. Loeser JD, Melzack R. Pain: an overview. *The Lancet.* 1999/05/08/ 1999;353(9164):1607-1609.

4. Besson JM. The neurobiology of pain. *The Lancet.* 1999/05/08/ 1999;353(9164):1610-1615.

5. Melzack R, Wall PD. Pain Mechanisms: A New Theory. *Science.* 1965;150(3699):971-979.

6. Melzack R, Katz J. Pain. *Wiley Interdisciplinary Reviews: Cognitive Science.* 2013;4(1):1-15.

7. Melzack R, Casey K. *Melzack & Casey Determinants of Pain 1968 from original*; 2014.

8. Besson JM, Chaouch A. Peripheral and spinal mechanisms of nociception. *Physiol Rev.* Jan 1987;67(1):67-186.

9. Merskey H, Bogduk N, Taxonomy IAftSoPTFo. *Classification of Chronic Pain: Descriptions of Chronic Pain Syndromes and Definitions of Pain Terms*: IASP Press; 1994.

10. Sherrington CS. *The Integrative Action of the Nervous System*: Yale University Press; 1906.

11. Tavares I, Martins I. *Gene Therapy for Chronic Pain Management*; 2013.

12. Ossipov MH, Dussor GO, Porreca F. Central modulation of pain. *J Clin Invest.* Nov 2010;120(11):3779-3787.

13. Dubin AE, Patapoutian A. Nociceptors: the sensors of the pain pathway. *The Journal of Clinical Investigation.* 11/01 2010;120(11):3760-3772.

14. Bingham B, Ajit SK, Blake DR, Samad TA. The molecular basis of pain and its clinical implications in rheumatology. *Nat Clin Pract Rheumatol.* Jan 2009;5(1):28-37.

15. Julius D, Basbaum AI. Molecular mechanisms of nociception. *Nature.* Sep 13 2001;413(6852):203-210.

16. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. *Cell.* Oct 16 2009;139(2):267-284.

17. Kuner R. Central mechanisms of pathological pain. *Nat Med.* 11//print 2010;16(11):1258-1266.

18. Grace PM, Hutchinson MR, Maier SF, Watkins LR. Pathological pain and the neuroimmune interface. *Nat Rev Immunol.* Apr 2014;14(4):217-231.

19. Ossipov MH, Lai J, King T, Vanderah TW, Porreca F. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers.* 2005;80(2-3):319-324.

20. Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci.* 1991;14:219-245.
21. Schaible HG. Peripheral and central mechanisms of pain generation. *Handb Exp Pharmacol.* 2007(177):3-28.
22. Mason P. Contributions of the medullary raphe and ventromedial reticular region to pain modulation and other homeostatic functions. *Annu Rev Neurosci.* 2001;24:737-777.
23. Foo H, Mason P. Brainstem modulation of pain during sleep and waking. *Sleep Med Rev.* Apr 2003;7(2):145-154.
24. Pertovaara A. The noradrenergic pain regulation system: a potential target for pain therapy. *Eur J Pharmacol.* Sep 15 2013;716(1-3):2-7.
25. Pertovaara A. Noradrenergic pain modulation. *Prog Neurobiol.* Oct 2006;80(2):53-83.
26. Llorca-Torralba M, Borges G, Neto F, Mico JA, Berrocoso E. Noradrenergic Locus Coeruleus pathways in pain modulation. *Neuroscience.* Dec 03 2016;338:93-113.
27. Dahlstrom A, Fuxe K. Localization of monoamines in the lower brain stem. *Experientia.* Jul 15 1964;20(7):398-399.
28. Halliday G. *Substantia Nigra and Locus Coeruleus*; 2004.
29. Ferrucci M, Giorgi FS, Bartalucci A, Busceti CL, Fornai F. The Effects of Locus Coeruleus and Norepinephrine in Methamphetamine Toxicity. *Current Neuropharmacology.* 01/
06/06/received
07/25/revised
08/08/accepted 2013;11(1):80-94.
30. Benarroch EE. Locus coeruleus. *Cell Tissue Res.* Jul 07 2017.
31. MacDonald E, Scheinin M. Distribution and pharmacology of alpha 2-adrenoceptors in the central nervous system. *J Physiol Pharmacol.* Sep 1995;46(3):241-258.
32. Berridge CW, Waterhouse BD. The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Brain Res Rev.* Apr 2003;42(1):33-84.
33. Atzori M, Cuevas-Olguin R, Esquivel-Rendon E, et al. Locus Ceruleus Norepinephrine Release: A Central Regulator of CNS Spatio-Temporal Activation? *Frontiers in Synaptic Neuroscience.* 08/26
06/12/received
08/05/accepted 2016;8:25.
34. Bajic D, Proudfit HK. Projections of neurons in the periaqueductal gray to pontine and medullary catecholamine cell groups involved in the modulation of nociception. *J Comp Neurol.* Mar 15 1999;405(3):359-379.

35. Cedarbaum JM, Aghajanian GK. Activation of locus coeruleus neurons by peripheral stimuli: modulation by a collateral inhibitory mechanism. *Life Sci.* Oct 02 1978;23(13):1383-1392.

36. Kuraishi Y, Hirota N, Sato Y, Kaneko S, Satoh M, Takagi H. Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. *Brain Res.* Dec 16 1985;359(1-2):177-182.

37. Jasmin L, Tien D, Weinshenker D, et al. The NK1 receptor mediates both the hyperalgesia and the resistance to morphine in mice lacking noradrenaline. *Proc Natl Acad Sci U S A.* Jan 22 2002;99(2):1029-1034.

38. Mansikka MDPDH, Lähdesmäki MDPDJ, Scheinin MDPDM, Pertovaara MDPDA. α2AAdrenoceptors Contribute to Feedback Inhibition of Capsaicin-induced Hyperalgesia. *Anesthesiology.* 2004;101(1):185-190.

39. Tsuruoka M, Willis WD. Bilateral lesions in the area of the nucleus locus coeruleus affect the development of hyperalgesia during carrageenan-induced inflammation. *Brain research.* 1996/07// 1996;726(1-2):233-236.

40. Muto Y, Sakai A, Sakamoto A, Suzuki H. Activation of NK(1) receptors in the locus coeruleus induces analgesia through noradrenergic-mediated descending inhibition in a rat model of neuropathic pain. *Br J Pharmacol.* Jun 2012;166(3):1047-1057.

41. Martins I, Carvalho P, de Vries MG, et al. Increased noradrenergic neurotransmission to a pain facilitatory area of the brain is implicated in facilitation of chronic pain. *Anesthesiology.* Sep 2015;123(3):642-653.

42. Martins I, Costa-Araujo S, Fadel J, Wilson SP, Lima D, Tavares I. Reversal of neuropathic pain by HSV-1-mediated decrease of noradrenaline in a pain facilitatory area of the brain. *Pain.* Oct 2010;151(1):137-145.

43. Martins I, de Vries MG, Teixeira-Pinto A, et al. Noradrenaline increases pain facilitation from the brain during inflammatory pain. *Neuropharmacology.* 2013/08/01/ 2013;71:299-307.

44. Doan L, Manders T, Wang J. Neuroplasticity Underlying the Comorbidity of Pain and Depression. *Neural Plasticity.* 02/25 12/05/received
02/10/accepted 2015;2015:504691.

45. Bair MJ, Robinson RL, Katon W, Kroenke K. Depression and pain comorbidity: A literature review. *Archives of Internal Medicine.* 2003;163(20):2433-2445.

46. Maletic V, Raison CL. Neurobiology of depression, fibromyalgia and neuropathic pain. *Front Biosci (Landmark Ed).* Jun 01 2009;14:5291-5338.

47. Doan L, Manders T, Wang J. Neuroplasticity underlying the comorbidity of pain and depression. *Neural Plast.* 2015;2015:504691.
48. Becerra L, Breiter HC, Wise R, Gonzalez RG, Borsook D. Reward circuitry activation by noxious thermal stimuli. *Neuron.* Dec 06 2001;32(5):927-946.
49. Gear RW, Levine JD. Nucleus accumbens facilitates nociception. *Exp Neurol.* Jun 2011;229(2):502-506.
50. Basbaum AI, Fields HL. Endogenous pain control mechanisms: review and hypothesis. *Ann Neurol.* Nov 1978;4(5):451-462.
51. Bushnell MC, Čeko M, Low LA. Cognitive and emotional control of pain and its disruption in chronic pain. *Nature reviews. Neuroscience.* 05/30 2013;14(7):502-511.
52. Alba-Delgado C, Llorca-Torralba M, Horrillo I, et al. Chronic pain leads to concomitant noradrenergic impairment and mood disorders. *Biol Psychiatry.* Jan 01 2013;73(1):54-62.
53. Yalcin I, Barrot M. The anxiodepressive comorbidity in chronic pain. *Curr Opin Anaesthesiol.* Oct 2014;27(5):520-527.
54. Borges G, Berrocoso E, Mico JA, Neto F. ERK1/2: Function, signaling and implication in pain and pain-related anxio-depressive disorders. *Prog Neuropsychopharmacol Biol Psychiatry.* Jul 03 2015;60:77-92.
55. Borges G, Neto F, Mico JA, Berrocoso E. Reversal of Monoarthritis-induced Affective Disorders by Diclofenac in Rats. *Anesthesiology.* 2014;120(6):1476-1490.
56. Borges GS, Berrocoso E, Ortega-Alvaro A, Mico JA, Neto FL. Extracellular signal-regulated kinase activation in the chronic constriction injury model of neuropathic pain in anaesthetized rats. *European journal of pain (London, England).* 2013/01// 2013;17(1):35-45.
57. Leadley RM, Armstrong N, Lee YC, Allen A, Kleijnen J. Chronic diseases in the European Union: the prevalence and health cost implications of chronic pain. *J Pain Palliat Care Pharmacother.* Dec 2012;26(4):310-325.
58. Torrance N, Smith BH, Bennett MI, Lee AJ. The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. *J Pain.* Apr 2006;7(4):281-289.
59. Bouhassira D, Lanteri-Minet M, Attal N, Laurent B, Touboul C. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain.* Jun 2008;136(3):380-387.
60. Treede RD, Jensen TS, Campbell JN, et al. Neuropathic pain: redefinition and a grading system for clinical and research purposes. *Neurology.* Apr 29 2008;70(18):1630-1635.
61. Colloca L, Ludman T, Bouhassira D, et al. Neuropathic pain. *Nature reviews. Disease primers.* 02/16 2017;3:17002-17002.

62. Watson JC, Sandroni P. Central Neuropathic Pain Syndromes. *Mayo Clin Proc.* Mar 2016;91(3):372-385.
63. Borsook D. Neurological diseases and pain. *Brain.* 11/08
03/03/received
08/18/revised
08/21/accepted 2012;135(2):320-344.
64. MacGowan DJ, Janal MN, Clark WC, et al. Central poststroke pain and Wallenberg's lateral medullary infarction: frequency, character, and determinants in 63 patients. *Neurology.* Jul 1997;49(1):120-125.
65. Jensen TS, Lenz FA. Central post-stroke pain: a challenge for the scientist and the clinician. *Pain.* May 1995;61(2):161-164.
66. Greenspan JD, Ohara S, Sarlani E, Lenz FA. Allodynia in patients with post-stroke central pain (CPSP) studied by statistical quantitative sensory testing within individuals. *Pain.* Jun 2004;109(3):357-366.
67. Finnerup NB, Haroutounian S, Kamerman P, et al. Neuropathic pain: an updated grading system for research and clinical practice. *Pain.* Aug 2016;157(8):1599-1606.
68. Baron R. Mechanisms of disease: neuropathic pain--a clinical perspective. *Nat Clin Pract Neurol.* Feb 2006;2(2):95-106.
69. Dworkin RH, O'Connor AB, Audette J, et al. Recommendations for the Pharmacological Management of Neuropathic Pain: An Overview and Literature Update. *Mayo Clinic Proceedings.* 2010;85(3 Suppl):S3-S14.
70. O'Connor AB. Neuropathic pain: quality-of-life impact, costs and cost effectiveness of therapy. *Pharmacoeconomics.* 2009;27(2):95-112.
71. Stillman M. Clinical approach to patients with neuropathic pain. *Cleve Clin J Med.* Aug 2006;73(8):726-728, 729-730, 733-726 passim.
72. O'Connor AB, Dworkin RH. Treatment of Neuropathic Pain: An Overview of Recent Guidelines. *The American Journal of Medicine.* 2009/10/01/ 2009;122(10, Supplement):S22-S32.
73. Finnerup NB, Otto M, McQuay HJ, Jensen TS, Sindrup SH. Algorithm for neuropathic pain treatment: an evidence based proposal. *Pain.* Dec 05 2005;118(3):289-305.
74. Jaggi AS, Jain V, Singh N. Animal models of neuropathic pain. *Fundam Clin Pharmacol.* Feb 2011;25(1):1-28.
75. Sorkin LS, Doom CM, Maruyama KP, Nanigian DB. Secondary hyperalgesia in the rat first degree burn model is independent of spinal cyclooxygenase and nitric oxide synthase. *European journal of pharmacology.* 04/01 2008;587(1-3):118-123.
76. Campbell JN, Meyer RA. Mechanisms of Neuropathic Pain. *Neuron.* 2006;52(1):77-92.
77. Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain.* Sep 1992;50(3):355-363.

78. Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain.* Nov 1990;43(2):205-218.
79. Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *PAIN.* 2000;87(2):149-158.
80. Molet J, Pohl M. Gene-based approaches in pain research and exploration of new therapeutic targets and strategies. *European Journal of Pharmacology.* 2013/09/15/ 2013;716(1):129-141.
81. Huang Y, Liu X, Dong L, Liu Z, He X, Liu W. Development of viral vectors for gene therapy for chronic pain. *Pain Res Treat.* 2011;2011:968218.
82. Gore BB, Soden ME, Zweifel LS. Manipulating gene expression in projection-specific neuronal populations using combinatorial viral approaches. *Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.].* 10/23 2013;4(435):4.35.31-34.35.20.
83. Kumar S, Ruchi R, James SR, Chidiac EJ. Gene Therapy for Chronic Neuropathic Pain: How Does It Work and Where Do We Stand Today? *Pain Medicine.* 2011;12(5):808-822.
84. Goins WF, Goss JR, Chancellor MB, de Groat WC, Glorioso JC, Yoshimura N. Herpes simplex virus vector-mediated gene delivery for the treatment of lower urinary tract pain. *Gene therapy.* 02/26 2009;16(4):558-569.
85. Dani SU. The challenge of vector development in gene therapy. *Brazilian Journal of Medical and Biological Research.* 1999;32.
86. Hao S, Mata M, Fink DJ. Viral vector-based gene transfer for treatment of chronic pain. *Int Anesthesiol Clin.* Spring 2007;45(2):59-71.
87. Yeomans DC, Lu Y, Laurito CE, et al. Recombinant herpes vector-mediated analgesia in a primate model of hyperalgesia. *Mol Ther.* Mar 2006;13(3):589-597.
88. Xu Y, Gu Y, Wu P, Li GW, Huang LY. Efficiencies of transgene expression in nociceptive neurons through different routes of delivery of adeno-associated viral vectors. *Hum Gene Ther.* Jun 10 2003;14(9):897-906.
89. Pohl M, Braz J. Gene therapy of pain: emerging strategies and future directions. *Eur J Pharmacol.* Oct 19 2001;429(1-3):39-48.
90. Benihoud K, Yeh P, Perricaudet M. Adenovirus vectors for gene delivery. *Curr Opin Biotechnol.* Oct 1999;10(5):440-447.
91. Federici T, Kutner R, Zhang XY, et al. Comparative analysis of HIV-1-based lentiviral vectors bearing lyssavirus glycoproteins for neuronal gene transfer. *Genet Vaccines Ther.* Jan 13 2009;7:1.
92. Manfredsson FP, Mandel RJ. Development of gene therapy for neurological disorders. *Discov Med.* Mar 2010;9(46):204-211.

93. Liu W, Liu Z, Cao X, et al. Recombinant human foamy virus, a novel vector for neurological disorders gene therapy, drives production of GAD in cultured astrocytes. *Mol Ther.* Oct 2007;15(10):1834-1841.
94. Howarth JL, Lee YB, Uney JB. Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ETCS-UK 1 day meeting on genetic manipulation of cells). *Cell Biol Toxicol.* Feb 2010;26(1):1-20.
95. Tavares I, Lima D. From neuroanatomy to gene therapy: searching for new ways to manipulate the supraspinal endogenous pain modulatory system. *Journal of Anatomy.* 03/20/accepted 2007;211(2):261-268.
96. Woo AKM. Depression and Anxiety in Pain. *Reviews in Pain.* 2010;4(1):8-12.
97. Feil S, Valtcheva N, Feil R. Inducible Cre mice. *Methods Mol Biol.* 2009;530:343-363.
98. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain.* Jun 1983;16(2):109-110.
99. Bourquin AF, Suveges M, Pertin M, et al. Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. *Pain.* May 2006;122(1-2):14.e11-14.
100. Curto-Reyes V, Kirschmann G, Pertin M, Drexler SK, Decosterd I, Suter MR. Neuropathic Pain Phenotype Does Not Involve the NLRP3 Inflammasome and Its End Product Interleukin-1β in the Mice Spared Nerve Injury Model. *PLOS ONE.* 2015;10(7):e0133707.
101. Erichsen HK, Hao J-X, Xu X-J, Blackburn-Munro G. A comparison of the antinociceptive effects of voltage-activated Na+ channel blockers in two rat models of neuropathic pain. *European Journal of Pharmacology.* 2003/01/05/ 2003;458(3):275-282.
102. Hao JX, Xu IS, Xu XJ, Wiesenfeld-Hallin Z. Effects of intrathecal morphine, clonidine and baclofen on allodynia after partial sciatic nerve injury in the rat. *Acta Anaesthesiologica Scandinavica.* 1999;43(10):1027-1034.
103. R. Bailey K, N. Crawley J. *Anxiety-Related Behaviors in Mice*; 2009.
104. Komada M, Takao K, Miyakawa T. Elevated Plus Maze for Mice. *Journal of Visualized Experiments : JoVE.* 12/22 2008(22):1088.
105. Deacon RMJ. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nat. Protocols.* 06//print 2006;1(1):122-124.
106. Angoa-Pérez M, Kane MJ, Briggs DI, Francescutti DM, Kuhn DM. Marble Burying and Nestlet Shredding as Tests of Repetitive, Compulsive-like Behaviors in Mice. *Journal of Visualized Experiments : JoVE.* 12/24 2013(82):50978.
107. Can A, Dao DT, Arad M, Terrillion CE, Piantadosi SC, Gould TD. The Mouse Forced Swim Test. *Journal of Visualized Experiments : JoVE.* 01/29 2012(59):3638.

108. Melzack R, Wall PD, Ty TC. Acute pain in an emergency clinic: latency of onset and descriptor patterns related to different injuries. *Pain.* Sep 1982;14(1):33-43.
109. Reynolds DV. Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science.* Apr 25 1969;164(3878):444-445.
110. Millan MJ. Descending control of pain. *Prog Neurobiol.* Apr 2002;66(6):355-474.
111. Jones SL. Descending noradrenergic influences on pain. *Prog Brain Res.* 1991;88:381-394.
112. Goncalves L, Silva R, Pinto-Ribeiro F, et al. Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. *Exp Neurol.* Sep 2008;213(1):48-56.
113. Suzuki T, Amata M, Sakaue G, et al. Experimental neuropathy in mice is associated with delayed behavioral changes related to anxiety and depression. *Anesth Analg.* Jun 2007;104(6):1570-1577, table of contents.
114. Yalcin B, Wong K, Agam A, et al. Sequence-based characterization of structural variation in the mouse genome. *Nature.* 09/15/print 2011;477(7364):326-329.
115. Wallace VCJ, Segerdahl AR, Blackbeard J, Pheby T, Rice ASC. Anxiety-like behaviour is attenuated by gabapentin, morphine and diazepam in a rodent model of HIV anti-retroviral-associated neuropathic pain. *Neuroscience Letters.* 07/23/received
09/30/revised
10/01/accepted 2008;448(1-9):153-156.
116. Hasnie FS, Breuer J, Parker S, et al. Further characterization of a rat model of varicella zoster virus-associated pain: Relationship between mechanical hypersensitivity and anxiety-related behavior, and the influence of analgesic drugs. *Neuroscience.* Feb 23 2007;144(4):1495-1508.
117. Mogil JS, Davis KD, Derbyshire SW. The necessity of animal models in pain research. *Pain.* Oct 2010;151(1):12-17.
118. Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT. Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology (Berl).* Sep 1994;116(1):56-64.
119. Paterson NE, Iwunze M, Davis SF, Malekiani SA, Hanania T. Comparison of the predictive validity of the mirror chamber and elevated plus maze tests in mice. *J Neurosci Methods.* Apr 30 2010;188(1):62-70.
120. De Boer SF, Koolhaas JM. Defensive burying in rodents: ethology, neurobiology and psychopharmacology. *Eur J Pharmacol.* Feb 28 2003;463(1-3):145-161.
121. Yankelevitch-Yahav R, Franko M, Huly A, Doron R. The Forced Swim Test as a Model of Depressive-like Behavior. *Journal of Visualized Experiments : JoVE.* 03/02 2015(97):52587.

122. Cryan JF, Valentino RJ, Lucki I. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neuroscience & Biobehavioral Reviews.* 2005/01/01/ 2005;29(4):547-569.
123. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther.* Oct 1977;229(2):327-336.
124. Narita M, Kuzumaki N, Narita M, et al. Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical δ-opioid receptor dysfunction. *Journal of Neurochemistry.* 2006;97(5):1369-1378.
125. Matsuzawa-Yanagida K, Narita M, Nakajima M, et al. Usefulness of Antidepressants for Improving the Neuropathic Pain-Like State and Pain-Induced Anxiety through Actions at Different Brain Sites. *Neuropsychopharmacology.* 10/24/online 2007;33(8):1952-1965.
126. Sorkin LS, Yaksh TL. Behavioral models of pain states evoked by physical injury to the peripheral nerve. *Neurotherapeutics.* 2009;6(4):609-619.
127. Crawley JN, Roth RH, Maas JW. Locus coeruleus stimulation increases noradrenergic metabolite levels in rat spinal cord. *Brain Res.* Apr 20 1979;166(1):180-184.
128. Hentall ID, Mesigil R, Pinzon A, Noga BR. Temporal and spatial profiles of pontine-evoked monoamine release in the rat's spinal cord. *J Neurophysiol.* Jun 2003;89(6):2943-2951.
129. Reddy SV, Maderdrut JL, Yaksh TL. Spinal cord pharmacology of adrenergic agonist-mediated antinociception. *J Pharmacol Exp Ther.* Jun 1980;213(3):525-533.
130. Hammond DL, Yaksh TL. Antagonism of stimulation-produced antinociception by intrathecal administration of methysergide or phentolamine. *Brain Res.* Apr 30 1984;298(2):329-337.
131. North RA, Yoshimura M. The actions of noradrenaline on neurones of the rat substantia gelatinosa in vitro. *J Physiol.* Apr 1984;349:43-55.
132. Jones SL, Gebhart GF. Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: mediation by spinal alpha 2-adrenoceptors. *Brain Res.* Feb 05 1986;364(2):315-330.
133. Miller JF, Proudfit HK. Antagonism of stimulation-produced antinociception from ventrolateral pontine sites by intrathecal administration of alpha-adrenergic antagonists and naloxone. *Brain Res.* Oct 15 1990;530(1):20-34.
134. Kawasaki Y, Kumamoto E, Furue H, Yoshimura M. Alpha 2 adrenoceptor-mediated presynaptic inhibition of primary afferent glutamatergic transmission in rat substantia gelatinosa neurons. *Anesthesiology.* Mar 2003;98(3):682-689.
135. Hughes SW, Hickey L, Hulse RP, Lumb BM, Pickering AE. Endogenous analgesic action of the pontospinal noradrenergic

system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury. *Pain.* Sep 2013;154(9):1680-1690.

136. Ma W, Eisenach JC. Chronic constriction injury of sciatic nerve induces the up-regulation of descending inhibitory noradrenergic innervation to the lumbar dorsal horn of mice. *Brain Res.* Apr 25 2003;970(1-2):110-118.

137. Hayashida K, Clayton BA, Johnson JE, Eisenach JC. Brain derived nerve growth factor induces spinal noradrenergic fiber sprouting and enhances clonidine analgesia following nerve injury in rats. *Pain.* Jun 2008;136(3):348-355.

138. Brightwell JJ, Taylor BK. Noradrenergic neurons in the locus coeruleus contribute to neuropathic pain. *Neuroscience.* Apr 21 2009;160(1):174-185.

139. Hirschberg S, Li Y, Randall A, Kremer EJ, Pickering AE. Functional dichotomy in spinal- vs prefrontal-projecting locus coeruleus modules splits descending noradrenergic analgesia from ascending aversion and anxiety in rats. *eLife.* 2017/10/13 2017;6:e29808.